AFRRI Reports

1990

January

February

March

Defense Nuclear Agency

Armed Forces Radiobiology Research Institute

Bethesda, Maryland 20814-5145

UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b RESTRICTIVE MARKINGS
2a SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited.
2b DECLASSIFICATION/DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S) SR90-1 - SR90-6	5 MONITORING ORGANIZATION REPORT NUMBER(S)
6a NAME OF PERFORMING ORGANIZATION Armed Forces Radiobiology Research Institute 6b OFFICE SYMBOL (If applicable) AFRRI	7a NAME OF MONITORING ORGANIZATION
6c ADDRESS (City, State, and ZIP Code) Defense Nuclear Agency Bethesda, Maryland 20814-5145	7b ADDRESS (City, State, and ZIP Code)
8a NAME OF FUNDING/SPONSORING ORGANIZATION Defense Nuclear Agency 8b OFFICE SYMBOL (If applicable) DNA	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c ADDRESS (City, State, and ZIP Code) Washington, DC 20305	10 SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO: NWED QAXM; PROJECT NO; TASK NO; WORK UNIT ACCESSION NO
11 TITLE (Include Security Classification) AFRRI Reports, Jan-Mar 1990	
12 PERSONAL AUTHOR(S)	
13a TYPE OF REPORT Reprints/Technical 13b. TIME COVERED FROM _____ TO _____	14. DATE OF REPORT (Year, Month, Day) 1990 April 15. PAGE COUNT 54
16 SUPPLEMENTARY NOTATION	
17 COSATI CODES FIELD / GROUP / SUB-GROUP	18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

This volume contains AFRRI Scientific Reports SR90-1 through SR90-6 for Jan-Mar 1990.

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL Gloria Ruggiero	22b. TELEPHONE (Include Area Code) (301) 295-2017 22c. OFFICE SYMBOL ISDP

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
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RADIATION RESEARCH 121, 338-343 (1990)

Hydrogen Peroxide-Induced Base Damage in Deoxyribonucleic Acid

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BLAKELY, W. F., FUCIARELLI, A. F., WEGHER, B. J., AND DIZDAROGLU, M. Hydrogen Peroxide-Induced Base Damage in Deoxyribonucleic Acid. *Radiat. Res.* 121, 338–343 (1990).

Aqueous solutions of calf thymus deoxyribonucleic acid (DNA) were exposed to hydrogen peroxide in the presence of air. Base products formed in DNA were identified and quantitated following acid hydrolysis and trimethylsilylation using gas chromatography–mass spectrometry. The yields of these products were dependent upon the hydrogen peroxide concentration, and increased in the following order: 8-hydroxyadenine, cytosine glycol, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 8-hydroxyguanine, thymine glycol, and 4,6-diamino-5-formamidopyrimidine. Previous studies have shown that these compounds are typically formed in DNA in aqueous solution by hydroxyl radicals generated by ionizing radiation. Hydrogen peroxide is thought to participate in a Fenton-like reaction with transition metals, which are readily bound to DNA in trace quantities, resulting in the production of hydroxyl radicals close to the DNA. This proposed mechanism was examined by exposing DNA to hydrogen peroxide either in the presence of a hydroxyl radical scavenger or following pretreatment of DNA with metal-ion chelators. The results indicate that trace quantities of transition metal ions can react readily with hydrogen peroxide to produce radical species. The production of radical species was monitored by determining the altered bases that resulted from the reaction between radicals and DNA. The yields of the base products were reduced by 40 to 60% with 10 mmol dm^{-3} of dimethyl sulfoxide. A 100-fold increase in the concentration of dimethyl sulfoxide did not result in a further reduction in hydrogen peroxide-induced base damage. DNA which was freed from bound metal ions by pretreatment with metal ion chelators followed by exhaustive dialysis was found to be an ineffective substrate for hydrogen peroxide. The yields of base products measured in this DNA were at background levels. These results support the role of metal ions bound to DNA in the site-specific formation of highly reactive radical species, most likely hydroxyl radicals, in hydrogen peroxide-induced damage to the bases in DNA.

INTRODUCTION

Hydrogen peroxide (H_2O_2) is ubiquitous in biological systems, formed as a consequence of a variety of metabolic processes. While normal cellular levels of H_2O_2 are generally low (i.e., 10^{-8} mol dm^{-3}) (*1*), these levels can be elevated as the result of inflammation (*2*), exposure to ionizing radiation (*3*), thiol oxidation (*4*), and metabolism of carcinogenic agents (*5, 6*). As a low-molecular-weight and uncharged species, H_2O_2 can diffuse readily from the site of production and traverse cell membranes (*7*).

In the presence of trace amounts of transition metal (M) ions (i.e., Fe^{2+}, Cu^{1+}) found in biological systems (*8*), H_2O_2 can participate readily in Fenton-like reactions (*9, 10*) resulting in the production of $^{\cdot}OH$ (see reaction below)

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^- + {}^{\cdot}OH.$$

Hydroxyl radicals have been implicated as the causative agent in deleterious processes such as gene mutation (*11*), cell transformation (*12*), and cell death (*13*). Hence recent interest has focused on examining the role of H_2O_2 in the induction of cellular injury and DNA damage (*14*). Current evidence suggests that under physiological conditions (i.e., pH 7) iron or copper ions interact with H_2O_2, forming a metal-peroxo complex composed of tetravalent (ferryl) iron or trivalent copper (*15, 16*). This metal-peroxo species, while not as thoroughly studied as $^{\cdot}OH$, appears to exhibit properties similar to $^{\cdot}OH$. Transition metal-hydrogen peroxide complexes may react directly with a substrate, as demonstrated under acidic conditions for Cu^{+} by Masarwa and colleagues (*15*). Alternatively, they may hydrolyze to produce $^{\cdot}OH$ under aqueous conditions as suggested by Whitburn (*17*), and demonstrated under acidic conditions for Cr^{2+} (*15*).

H_2O_2-induced degradation of DNA *in vitro* and *in vivo* has been demonstrated by decreases in DNA viscosity (*18*), increases in both single-strand (*18–23*) and double-strand DNA breaks (*18*), formation of ammonia and inorganic phosphate (*24*), and base release (*25*). In addition to these alterations within the sugar phosphate backbone of DNA, H_2O_2 or its reactive radical byproducts are reported to react with DNA bases, resulting in both the loss of ultraviolet light absorbance (*18*) and the formation of altered bases (*6, 26–28*). However, the yield of H_2O_2-induced base modification has been shown to predominate over that of single-strand break formation in DNA by ~20- to 60-fold as mea-

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0033-7587/90 $3.00

sured by decreases in absorbance at 260 nm (*18*) and by the formation of 5,6-saturated thymine products (*27*).

In this investigation, methodology incorporating gas chromatography–mass spectrometry with selected ion monitoring (GC-MS/SIM) was used to reveal a wide spectrum of base products in DNA, which result from H_2O_2 exposure (*29*), and which are characteristic of those produced in DNA upon exposure to ionizing radiation (*30, 31*). In addition, studies were performed to examine the mechanism of H_2O_2-induced damage to DNA, which has been suggested to involve a site-specific reaction (*3, 32, 33*) catalyzed by trace quantities of transition metal ions bound to DNA (*34*).

MATERIALS AND METHODS[2]

Materials Hydrogen peroxide, calf thymus DNA, and ultra-pure calf thymus DNA were obtained from Sigma Chemical Co. Spectrapor dialysis membranes were purchased from Arthur H. Thomas Co. Water purified through a Millipore-Q system was used for all purposes. Acetonitrile and bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane were purchased from Pierce Chemical Co. Dimethyl sulfoxide (DMSO) was obtained from Eastman and was >99% pure. Metal ion chelators, which were of highest purity, were ethylenediaminetetraacetic acid (EDTA) (International Biotechnologies, Inc.), diethylenetriaminepentaacetic acid (DETAPAC) (Aldrich), and deferoxamine mesylate (desferal) (Ciba-Geigy Corp.).

H_2O_2 exposure Native calf thymus DNA was dissolved in 30 mmol dm^{-3} phosphate buffer (pH 7.2) at a final concentration of ~0.5 mg cm^{-3}. In designated experiments the metal ions present in the DNA solutions were chelated by treatment for 3 h at room temperature with EDTA, DETAPAC, or desferal at a final chelator concentration of 1 mmol dm^{-3}. Chelators are effective scavengers of radical species (*35*), and hence were removed along with the chelator–metal ion complexes by exhaustive dialysis using ~10^4 molecular weight cutoff membranes with 30 mmol dm^{-3} phosphate buffer (pH 7.2) prior to H_2O_2 exposures. Complete removal of the chelators was confirmed by uv spectral measurements of the resultant DNA solution.

Solutions of native DNA (0.5 mg cm^{-3}) were exposed to designated concentrations of H_2O_2 while saturated with air. The reaction of H_2O_2 with DNA was terminated by the addition of catalase (Worthington Biochemical Corp., Cat. No. 1896).

Sample preparation for GC-MS/SIM analysis The protocol and conditions used to prepare samples for GC-MS/SIM analysis were described previously (*31*). Briefly, H_2O_2-treated and control DNA solutions were dialyzed exhaustively against water to remove phosphate buffer salts using ~10^4 molecular weight cutoff membranes. Following dialysis, the DNA concentration for each aliquot was determined by uv absorbance measurements at 260 nm or by Burton's assay (*36*) as described below. Aliquots of the remaining solutions were then lyophilized. Internal standards (6-azathymine and 8-azaadenine) were added to the dried samples, which were then hydrolyzed with 1 ml of 88% formic acid (150°C, 40 min). The hydrolysate was dried and then trimethylsilylated in PTFE-capped Hypovials (Pierce Chemical Co.) with 0.2 ml of BSTFA/acetonitrile (3/1, v/v) mixture for 30 min at 130°C.

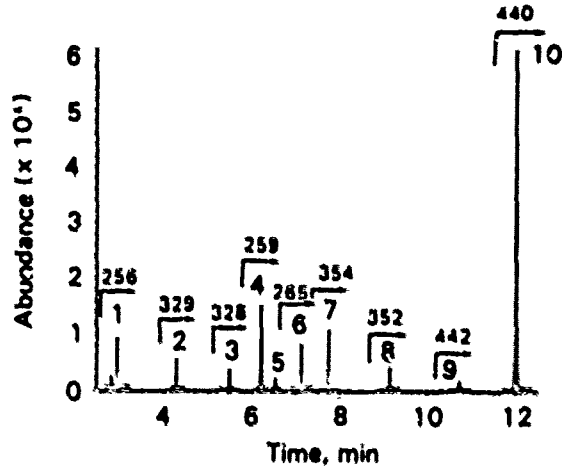

FIG. 1. Selected-ion chromatogram of a mixture of trimethylsilylated purine and pyrimidine products resulting from the exposure of DNA to H_2O_2 for 24 h. The ion monitored for each product over the time (min) in which that product elutes is shown above each peak. Peak identification is given in Table I.

GC-MS/SIM analysis All measurements were performed on a Hewlett-Packard Model 5970B mass selective detector controlled by a Hewlett-Packard Model 5997C computer work station and interfaced to a Hewlett-Packard 5890A gas chromatograph. The injection port and the ion source were kept at 250°C. The GC-MS interface was maintained at 270°C. Separations were carried out on a fused silica capillary column (12.5 m × 0.32 mm) coated with crosslinked 5% phenyl methyl silicone gum phase (film thickness, 0.17 μm) (Hewlett-Packard). The column temperature was increased from 120 to 250°C at a rate of 8°C per min after 2 min at 120°C. Helium was used as the carrier gas at an inlet pressure of 5 kPa. Samples were injected using the split mode. Mass spectra were obtained at 70 eV.

Measurement of product yields Product yields were determined as described previously (*31*). Briefly, quantitation of base damage products required accurate measurement of the amount of altered base present relative to DNA content. The amount of DNA present in each sample was determined by uv absorbance at 260 nm or by Burton's assay (*36*). The amount of DNA was similar in both measurements.

Quantitation of the amount of purine and pyrimidine product detected incorporated the use of internal standards. Relative molar response factors were obtained using these internal standards and authentic pure standards of the various products (*31*). Since acidic hydrolysis causes isomerization of *cis*-thymine glycol to *trans*-thymine glycol (*37*), the yields reported for these products represent the sum of *cis*- and *trans*-thymine glycol. The yields of cytosine glycol were determined as the sum of 5-hydroxyuracil and 5-hydroxycytosine as described previously (*37*).

RESULTS

H_2O_2-induced pyrimidine and purine products in DNA. Exposure of DNA to H_2O_2 in air-saturated solutions resulted in the formation of several purine and pyrimidine products. Figure 1 illustrates the ion-current profiles obtained during a GC-MS/SIM analysis of the trimethylsilylated hydrolysate of DNA treated with H_2O_2. The products identified were as follows: *cis*- and *trans*-5,6-dihydroxy-5,6-dihydrothymine (thymine glycol); 5,6-dihy-

[2] Mention of commercial products does not imply recommendation or endorsement by the Armed Forces Radiobiology Research Institute or the National Institute of Standards and Technology, nor does it imply that products identified are necessarily the best available for the purpose.

TABLE I
Peak Identification in Fig. 1

Peak	Compound[a]	Peak	Compound[a]
1	6-Azathymine	6	8-Azaadenine
2	5-Hydroxyuracil[b]	7	FAPy-adenine
3	5-Hydroxycytosine[c]	8	8-Hydroxyadenine
4	(cis) Thymine glycol	9	FAPy-guanine
5	(trans) Thymine glycol	10	8-Hydroxyguanine

[a] As their trimethylsilyl derivatives.
[b] 5-Hydroxyuracil is a product of the acid-induced deamination and dehydration of cytosine glycol.
[c] 5-Hydroxycytosine is a product of the acid-induced dehydration of cytosine glycol.

droxy-5,6-dihydrocytosine (cytosine glycol) [which is represented by 5-hydroxycytosine and 5-hydroxyuracil (*37*)]; 4,6-diamino-5-formamidopyrimidine (FAPy-adenine); 6-amino-8-hydroxypurine (8-hydroxyadenine); 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FAPy-guanine); and 2-amino-6,8-dihydroxypurine (8-hydroxyguanine). Two additional peaks corresponding to the internal standards, 6-azathymine and 8-azaadenine, are also illustrated in Fig. 1. (See Table I for peak identification in Fig. 1.)

The purine and pyrimidine products illustrated in Fig. 1 were also detected in untreated DNA samples, however, their yields were relatively low, compared to those found in DNA exposed for 24 h to concentrations of H_2O_2 in the range of 0.1 to 0.4 mol dm^{-3}. Figure 2A illustrates that the yields of pyrimidine glycols increased linearly with H_2O_2 concentration. The ratio of the yield of thymine glycol to

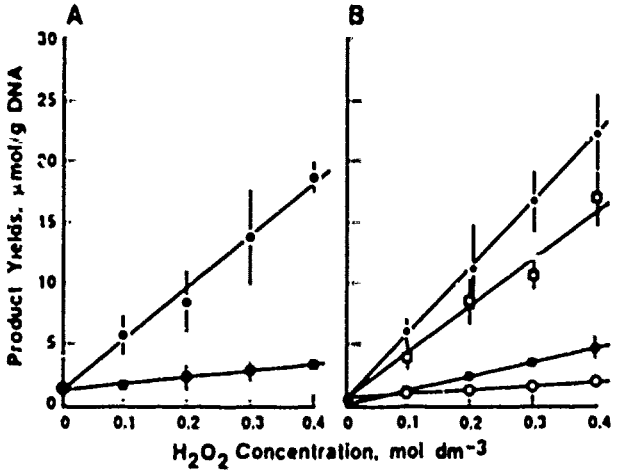

FIG. 2. Product formation in air-saturated solutions of native DNA exposed to H_2O_2 for 24 h. A, pyrimidine products. (●) thymine glycol, (■) cytosine glycol. B, purine products. (●) FAPy-adenine, (□) 8-hydroxyguanine, (■) FAPy-guanine, (○), 8-hydroxyadenine. Each data point represents the mean ± standard error from three replicate experiments; the data were fitted by least-squares linear regression analysis.

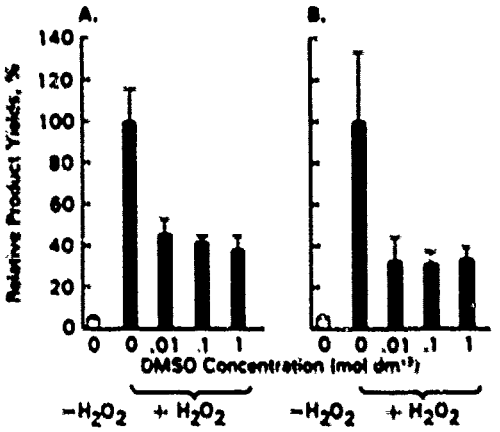

FIG. 3. The effect of dimethyl sulfoxide on H_2O_2-induced DNA base damage. The relative yields of thymine glycol (A) and 8-hydroxyguanine (B) following exposure of DNA to the following conditions: untreated (control) and 24 h H_2O_2 (400 mmol dm^{-3}) with the designated concentrations of DMSO. Each data point represents the mean (± standard error) from three replicate experiments.

that of cytosine glycol was 6.4 ± 0.22. The yield of the purine products was also linearly related to H_2O_2 concentration. The yield of FAPy-adenine was 16 ± 1.3-fold higher than that of 8-hydroxyadenine. This pattern was reversed for guanine products, for which the ratio of the yield of 8-hydroxyguanine to that of FAPy-guanine was 3.4 ± 0.28 (Fig. 2B).

Exposure to 0.4 mol dm^{-3} H_2O_2 for 24 h resulted in ~21 altered bases per 10^5 nucleotides of DNA, which represent less than 2.5% of the DNA nucleotides available. Correcting for the known base composition of calf thymus DNA (*38*), the relative susceptibility of the DNA bases to modification by exposure to H_2O_2 was as follows: cytosine (5.4%) < thymine (24.4%) < adenine (32.3%) < guanine (38%).

Effect of 'OH scavenger. The effect of DMSO, a hydroxyl radical scavenger, on H_2O_2-induced base damage was examined. When DNA was treated with H_2O_2 in the presence of 10 mmol dm^{-3} DMSO, there was a 40 to 60% reduction in all products measured. Further increases in DMSO concentration did not reduce the yields of these products. As an example, this effect is illustrated in Fig. 3 for thymine glycol and 8-hydroxyguanine.

Effect of pretreating DNA with chelators. The influence of DNA-bound metal ions on H_2O_2-induced base damage was investigated by examining the effect of DNA pretreatment with transition metal ion chelators. In these experiments, chelator-treated DNA solutions were dialyzed exhaustively to remove low-molecular-weight compounds, including the metal ion–chelator complexes. Pretreatment of DNA with desferal, EDTA, or DETAPAC inhibited formation of purine and pyrimidine products in H_2O_2-treated DNA. Figure 4 illustrates these results for thymine glycol and 8-hydroxyguanine.

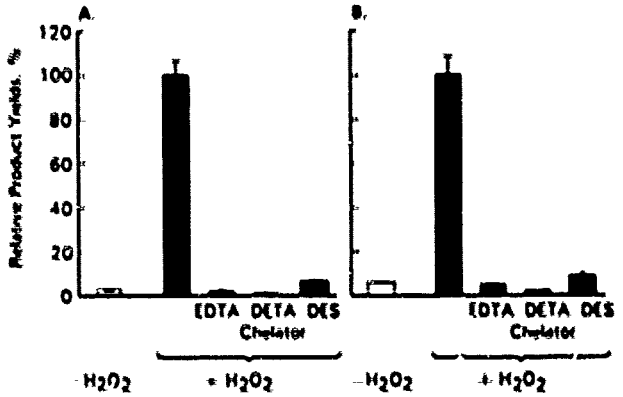

FIG. 4. The effect of pretreatment of DNA with the metal ion chelators EDTA, DETAPAC (DETA), and desferal (DES) on the relative yields of thymine glycol (A) and 8-hydroxyguanine (B). The yields of products in untreated (control) DNA are shown; all other samples were treated with H_2O_2 following pretreatment with the designated chelator (1 mmol dm^{-3}). Each data point represents the mean (± standard error) from three replicate experiments.

DISCUSSION

Hydrogen peroxide participates in a Fenton-type reaction with transition metal ions (e.g., iron and copper), which are readily bound to DNA in trace quantities [for a review see Izatt *et al.* (*34*)]. Hence Fenton reaction-produced hydroxyl radicals (*39*) formed in this manner is close to reactive centers in the purine and pyrimidine moieties of DNA. Reactions of this type that result in DNA damage have been characterized as exhibiting a "site-specific" nature (*3, 32, 33*). This study provides the first detailed comparison of the major DNA base products formed following H_2O_2 exposure of DNA in aqueous solution with no added metal ions. The pattern of the products observed was similar to that of base products identified following the exposure of DNA in aqueous solution to ionizing radiation (*3, 30, 31, 40*) or to superoxide radical-generating systems (*41, 42*). In the present work the likelihood of the involvement of $^{\cdot}OH$ in product formation is strongly supported by the ability of DMSO to reduce the yields of H_2O_2-induced base products significantly.

Dimethyl sulfoxide has been shown to reduce the yield of H_2O_2-induced single-strand breaks in DNA *in vitro* (*20, 21*) as well as DNA *in vivo* (*23*). Lesko *et al.* (*43*) demonstrated that DMSO was also effective at inhibiting Fe^{2+}–EDTA/H_2O_2-induced DNA–protein and DNA–intrastrand cross-linking in isolated chromatin. The results of this present study extend these findings by including the individual products of all four bases in DNA. However, in contrast to DMSO's ability to inhibit ≥90% of DNA single-strand break formation in isolated DNA exposed to H_2O_2 (*20, 21*), we found an inhibition of product formation by 40 to 60%, even at the highest scavenger concentration (1 mol dm^{-3}). This finding is consistent with a failure of the radical scavenger, DMSO, to react with radicals formed at the location of the metal ion-mediated site-specific reaction.

The reduced yields of H_2O_2-induced products to background levels in DNA, which were freed from metal ions by treatment with EDTA, DETAPAC, or desferal via exhaustive dialysis prior to H_2O_2 treatment, also strongly support the concept of a site-specific reaction. This also indicates that H_2O_2 alone has no measurable reactivity with DNA, in terms of the base products measured in this study. Inhibition of H_2O_2-induced single-strand breaks in DNA via pretreatment of DNA with various chelators has also been demonstrated using plasmid DNA (*20*) and isolated nuclei (*19, 22*). Our results agree with these findings and support the hypothesis that chelators eliminate an essential factor, presumably trace quantities of metal ions, required to elicit H_2O_2-induced damage to DNA bases.

Lesko *et al.* (*20*) argued that the effectiveness of the chelators in inhibiting DNA strand breaks induced by oxygen species was correlated to their chelating ability. In this study, high concentrations of the chelators were used. Hence marked differences in the effectiveness of the chelators were not observed.

In summary, the mechanism of H_2O_2-induced DNA damage appears to involve a role for transition metal ions bound to DNA interacting with H_2O_2, resulting in the production of a reactive radical species, most likely $^{\cdot}OH$. This radical species formed close to the DNA interacts with DNA, forming purine and pyrimidine products characteristic of those found after the exposure of aqueous DNA solutions to ionizing radiation. These results may help in investigations of enzymatic repair of base damage using a substrate with enriched yields of these altered bases (*44, 45*). In addition, similar measurements have been performed on DNA following exposure to ionizing radiation,[3] which will permit a direct comparison of product yield ratios.

ACKNOWLEDGMENTS

This work has been supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under Research Work Unit Numbers 00105 and 00150. Views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred. The authors would like to thank Drs. G. Czapski and J. F. Ward for their helpful discussions. In addition, we gratefully acknowledge Prof. Dr. D. Schulte-Frohlinde for his helpful review of this manuscript during its preparation.

RECEIVED: August 8, 1989; ACCEPTED: October 6, 1989

REFERENCES

1. A. BOVERIS and B. CHANCE, The mitochondrial generation of hydrogen peroxide. *Biochem. J.* 134, 707–716 (1973).

[3] A. F. Fuciarelli, B. J. Wegher, W. F. Blakely, and M. Dizdaroglu, Yields of radiation-induced base products in DNA: Effect of DNA conformation and gassing conditions. Manuscript submitted for publication.

2 J. W. T. HOMAN-MÜLLER, R. S. WEENING, and D. ROOS, Production of hydrogen peroxide by phagocytizing human granulocytes. *J Lab Clin Med* 85, 198–207 (1975).

3 C. VON SONNTAG, *The Chemical Basis of Radiation Biology*, Taylor & Francis, London, 1987.

4 J. E. BIAGLOW, R. W. ISSELS, L. E. GERWECK, M. E. VARNES, B. JACOBSON, J. B. MITCHELL, and A. RUSSO, Factors influencing the oxidation of cysteamine and other thiols: Implications for hyperthermic sensitization and radiation protection. *Radiat Res* 100, 298–312 (1984).

5 R. J. LORENTZEN and P. O. P. TSO, Benzo[a]pyrenedione/benzo[a]-pyrenediol oxidation-reduction couples and the generation of reactive reduced molecular oxygen. *Biochemistry* 16, 1467–1472 (1977).

6 J. G. LEWIS and D. O. ADAMS, Induction of 5,6-ring-saturated thymine bases in NIH-3T3 cells by phorbol ester-stimulated macrophages: Role of reactive oxygen intermediates. *Cancer Res* 45, 1270–1275 (1985).

7 A. A. FRIMER, A. FORMAN, and D. C. BORG, H_2O_2-diffusion through liposomes. *Int J Chem* 23, 442–445 (1983).

8 B. HALLIWELL and J. M. C. GUTTERIDGE, The importance of free radicals and catalytic metal ions in human diseases. *Mol. Aspects Med* 8, 89–193 (1985).

9 H. J. H. FENTON, Oxidation of tartaric acid in the presence of iron. *Chem Soc J* 65, 899–910 (1894).

10 G. COHEN, The Fenton reaction. In *CRC Handbook of Methods for Oxygen Radical Research* (R. A. Greenwald, Ed.), pp. 55–64. CRC Press, Boca Raton, FL, 1985.

11 S. OKADA, *Radiation Biochemistry*, Vol. I. Academic Press, New York, 1970.

12 C. BOREK, Induction and control of radiogenic transformations *in vitro*: cellular and molecular mechanisms. *Pharmac Ther* 27, 99–142 (1985).

13 R. B. PAINTER, The role of DNA damage and repair in cell killing induced by ionizing radiation. In *Radiation Biology in Cancer Research* (R. E. Meyn and H. R. Withers, Eds.), pp. 59–68. Raven Press, New York, 1980.

14 J. A. IMLAY and S. LINN, DNA damage and oxygen radical toxicity. *Science* 240, 1302–1309 (1988).

15 M. MAHARWA, H. COHEN, D. MEYERSTEIN, D. L. HUGKMAN, A. BAKAC, and J. H. ESPENSON, Reactions of low-valent transition-metal complexes with hydrogen peroxide. Are they "Fenton-like" or not? 1. The case of Cu^{+}_{aq} and Cr^{2+}_{aq}. *J Am Chem Soc* 110, 4293–4297 (1988).

16 H. C. SUTTON and C. C. WINTERBOURN, On the participation of higher states of iron and copper in Fenton reactions. *Free Radicals Biol Med* 6, 53–60 (1989).

17 K. D. WELTBURN, The interaction of oxymyoglobin with hydrogen peroxide: The formation of ferrylmyoglobin at moderate excesses of hydrogen peroxide. *Arch Biochem Biophys* 253, 419–430 (1987).

18 H. R. MASSIE, H. V. SAMIS, and M. B. BAIRD, The kinetics of degradation of DNA and RNA by H_2O_2. *Biochim Biophys Acta* 272, 539–548 (1972).

19 R. MENTGHINI and M. E. HOFFMANN, The damaging action of hydrogen peroxide on DNA of human fibroblasts is mediated by a non-dialyzable compound. *Biochim Biophys Acta* 608, 167–173 (1980).

20 S. A. LESKO, R. J. LORENTZEN, and P. O. P. TSO, Role of superoxide in deoxyribonucleic acid strand scission. *Biochemistry* 19, 3023–3028 (1980).

21 J. E. REPINE, O. W. PETTINGER, D. W. TALMAGE, E. M. BERGER, and D. E. PETTIJOHN, Dimethyl sulfoxide prevents DNA nicking by ionizing radiation or iron/hydrogen peroxide-generated hydroxyl radical. *Proc Natl Acad Sci USA* 78, 1001–1003 (1981).

22 A. C. MELLO FILHO and R. MENEGHINI, *In vivo* formation of single-strand breaks in DNA by hydrogen peroxide is mediated by the Haber-Weiss reaction. *Biochim. Biophys. Acta* 781, 56–63 (1984).

23 J. F. WARD, W. F. BLAKELY, and E. I. JONER, Mammalian cells are not killed by DNA single-strand breaks caused by hydroxyl radicals from hydrogen peroxide. *Radiat Res* 103, 383–392 (1985).

24 G. SCHOLES and J. WEISS, Chemical action of x-rays on nucleic acids and related substances in aqueous systems. *Biochem J* 53, 567–578 (1953).

25 H.-J. RHAESE and E. FREESE, Chemical analysis of DNA alterations, I. Base liberation and backbone breakage of DNA and oligodeoxyadenylic acid induced by hydrogen peroxide and hydroxylamine. *Biochim Biophys Acta* 155, 476–490 (1968).

26 H.-J. RHAESE, E. FREESE, and M. S. MELZER, Chemical analysis of DNA alterations. II. Alterations and liberation of bases of deoxynucleotides and deoxynucleosides induced by hydrogen peroxide and hydroxylamine. *Biochim. Biophys. Acta* 155, 491–504 (1968).

27 B. DEMPLE and S. LINN, 5,6-Saturated thymine lesions in DNA: Production by ultraviolet light or hydrogen peroxide. *Nucleic Acids Res* 10, 3781–3789 (1982).

28 H. KASAI and S. NISHIMURA, Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucleic Acids Res* 12, 2137–2145 (1984).

29 W. F. BLAKELY, E. HOLWITT and M. DIZDAROGLU, Characterization of H_2O_2-induced damage in DNA. In *Radiation Research* (E. M. Fielden, J. F. Fowler, J. H. Hendry, and D. Scott, Eds.), Vol. 1, p. 13. Taylor and Francis, London, 1987. [Abstract]

30 M. DIZDAROGLU, Application of capillary gas chromatography-mass spectrometry to chemical characterization of radiation-induced base damage of DNA: Implications for assessing DNA repair processes. *Anal Biochem* 144, 593–603 (1985).

31 A. F. FUCIARELLI, B. J. WEGHER, E. GAJEWSKI, M. DIZDAROGLU, and W. F. BLAKELY, Quantitative measurement of radiation-induced base products in DNA using gas chromatography-mass spectrometry. *Radiat Res* 119, 219–231 (1989).

32 A. SAMUNI, J. ARONOVITCH, D. GODINGER, M. CHEVION, and G. CZAPSKI, On the cytotoxicity of vitamin C and metal ions. A site-specific Fenton mechanism. *Eur J Biochem* 137, 119–124 (1933).

33 J. M. C. GUTTERIDGE, Copper-phenanthroline-induced site-specific oxygen-radical damage to DNA. Detection of loosely bound copper in biological fluids. *Biochem J* 218, 983–985 (1984).

34 R. M. IZATT, J. J. CHRISTENSEN, and J. H. RYTTING, Sites and thermodynamic quantities associated with proton and metal ion interaction with ribonucleic acid, deoxyribonucleic acid, and their constituent bases, nucleosides, and nucleotides *Chem Rev* 71, 439–481 (1971).

35 B. HALLIWELL, Use of desferrioxamine as a "probe" for iron-dependent formation of hydroxyl radicals. Evidence for a direct reaction between desferal and the superoxide radical. *Biochem. Pharmacol.* 34, 229–233 (1985).

36 K. BURTON, Determination of DNA concentration with diphenylamine. In *Methods in Enzymology*, Vol. 12, *Nucleic Acids* (L. Grossman and K. Moldaire, Eds.) Part B, pp. 163–166. Academic Press, New York, 1968.

37 M. DIZDAROGLU, E. HOLWITT, M. P. HAGAN, and W. F. BLAKELY, Formation of cytosine glycol and 5,6-dihydroxycytosine in deoxyribonucleic acid on treatment with osmium tetroxide. *Biochem. J.* 235, 531–536 (1986).

38 R. L. P. ADAMS, R. H. BURDON, A. M. CAMPBELL, and R. M. S. SMELLIE, *Davidson's The Biochemistry of the Nucleic Acids* Eighth ed. Academic Press, New York, 1976.

39 R. A. FLOYD, DNA-ferrous iron catalyzed hydroxyl free radical formation from hydrogen peroxide, *Biochem. Biophys. Res. Commun.* 99, 1209–1215 (1981).

40 R. HOCHE, Radiation-induced DNA damage and its repair, *Int. J. Radiat. Biol.* 51, 573–589 (1987).

41 O. I. APTSIAURI, B. HALLIWELL, and M. DIZDAROGLU, Iron ion-dependent modification of bases in DNA by the superoxide radical-generating system hypoxanthine/xanthine oxidase, *J. Biol. Chem.* 264, 13024–13028 (1989).

42 O. I. APTSIAURI, B. HALLIWELL, E. GAJEWSKI, and M. DIZDAROGLU, Damage to the bases in DNA induced by hydrogen peroxide and ferric ion chelates, *J. Biol. Chem.* 264, 20,509–20,512 (1989).

43 S. A. LESKO, J.-L. DROCOURT, and S.-U. YANG, Deoxyribonucleic acid-protein and deoxyribonucleic acid interstrand cross-links induced in isolated chromatin by hydrogen peroxide and ferrous ethylenediaminetetraacetate chelates, *Biochemistry* 21, 5010–5015 (1982).

44 I. H. BREIMER, Enzymatic excision from γ-irradiated polydeoxyribonucleotides of adenine whose imidazole rings have been ruptured, *Nucleic Acids Res.* 12, 6359–6367 (1984).

45 D. E. HELLAND, P. W. DOETSCH, and W. A. HASELTINE, Substrate specificity of a mammalian DNA repair endonuclease that recognizes oxidative base damage, *Mol. Cell. Biol.* 6, 1983–1990 (1986).

RADIATION RESEARCH 121, 169–174 (1990)

Reduction of 3-Methoxytyramine Concentrations in the Caudate Nucleus of Rats after Exposure to High-Energy Iron Particles: Evidence for Deficits in Dopaminergic Neurons

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HUNT, W. A., DALTON, T. K., JOSEPH, J. A., AND RABIN, B. M. Reduction of 3-Methoxytyramine Concentrations in the Caudate Nucleus of Rats after Exposure to High-Energy Iron Particles: Evidence for Deficits in Dopaminergic Neurons. *Radiat. Res.* 121, 169–174 (1990).

Exposure to low doses of high-energy iron particles can alter motor behavior. The ability of rats to hang from a wire has been reported to be significantly degraded after exposure to doses as low as 0.5 Gy. In addition, deficits in the ability of acetylcholine to regulate dopamine release in the caudate nucleus (an area in the brain important for motor function) have been found. The concentrations of 3-methoxytyramine (3-MT), a metabolite of dopamine whose concentrations reflect dopamine release *in vivo*, were measured after rats were exposed to different doses of high-energy iron particles to gain further information about the effect of radiation on the dopaminergic system. Concentrations of 3-MT were significantly reduced 3 days after exposure to 5 Gy but returned to control values by 8 days. After 6 months, concentrations were again less than control values. Exposure to 5 Gy of high-energy electrons or γ photons had no effect 3 days after exposure. Very high doses of electrons were needed to alter 3-MT concentrations. One hundred grays of electrons decreased 3-MT 30 min after irradiation but levels returned to control values by 60 min. Gamma photons had no effect after doses up to 200 Gy. These results provide further evidence that exposure to heavy particles can degrade motor behavior through an action on dopaminergic mechanisms and that this can occur after doses much lower than those needed for low-LET radiation.

INTRODUCTION

The prospect of long-term space travel raises a number of questions about the safety of astronauts asked to venture on prolonged journeys. The problems of microgravity are well known, but the hazards of exposure to radiation are less understood. Most space travel has involved spending a few days to many months in low-altitude, equatorial orbits, where the dangers of radiation are lessened by the magnetic field surrounding the earth.

Travel to polar or geostationary orbits or travel to the moon or the planets has a far greater radiation hazard. Although numerous studies have appeared which address the life-shortening possibilities and genetic defects that can result from exposure to radiations found in space, almost nothing is known about possible risks to behavior and brain function after radiation exposure, such as found after the emission of solar flares or from long-term exposure from galactic cosmic radiation. It is possible that the performance of important tasks by astronauts on a spacecraft may be progressively degraded under these circumstances and that their ability to survive may be compromised.

Solar flares are composed mostly of high-energy protons (*1*). However, during major solar events, the flux of heavy particles can rapidly increase by 3 or 4 orders of magnitude above the galactic cosmic-ray background (*2*). Also, heavy charged particles constitute about 1% of the galactic cosmic radiation with energies ranging to an excess of 10 GeV/nucleon. The importance of heavy charged particles in spite of their low fluence compared to protons is the problem of shielding. Curtis and Wilkinson (*3*) have calculated that as much as 10 g/cm^2 of aluminum can reduce the absorbed dose of heavy particles by only 20–40%. Consequently, the possible deleterious effects of exposure to these particles cannot be ignored.

Recent efforts by this laboratory have investigated the effect of heavy particles on behavior and neural function in rats and have revealed surprising results. Exposure to high-energy iron particles induced behavioral changes and neurochemical deficits that were at least 10 times greater than those found after exposure to γ photons. For example, the acquisition of a conditioned taste aversion (a general measure of behavioral toxicity (*4*)) and degradation in performance on a wire suspension task (a measure of upper body strength (*5*)) was apparent after doses as low as 0.1–0.2 Gy with maximal effects observed after 0.50 Gy (*6, 7*). Equivalent responses after γ irradiation required at least 5 Gy.

The deficits in performance on the wire suspension task were correlated with a reduced ability of acetylcholine to regulate the release of dopamine *in vitro* in the caudate nucleus (an area of the brain important in coordinating movement). Oxotremorine, an acetylcholine agonist, was used to enhance potassium-stimulated dopamine release from

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slices of the caudate nucleus. The release of dopamine was inhibited by 50% 3 days after exposure to 0.5 Gy of iron particles (*7*). However, a dose of 5 Gy had no effect 3 days after exposure on the performance on the wire suspension task or on dopamine release. But, 14 days after exposure, deficits were found in both end points. Possibly, other factors were playing a role in the behavioral and neural responses observed 3 days after exposure to the higher dose.

Since striatal function appears to be altered after exposure to iron particles, the present experiments were conducted to assess other possible abnormalities in dopaminergic activity by studying the metabolism of dopamine *in vivo*. The concentrations of the dopamine metabolites dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxytyramine (3-MT) have been used as an index of the rate of utilization of dopamine by neurons that use it as a neurotransmitter (*8*). The concentration of 3-MT, an O-methylated metabolite of dopamine, in particular, has been shown to correlate well with dopamine release (*9, 10*). In the present experiments, 3-MT concentrations as well as DOPAC and HVA were determined after various doses of high-energy iron particles and at different times after irradiation. For comparison, appropriate parallel measurements were obtained from animals exposed to high-energy electrons or γ photons.

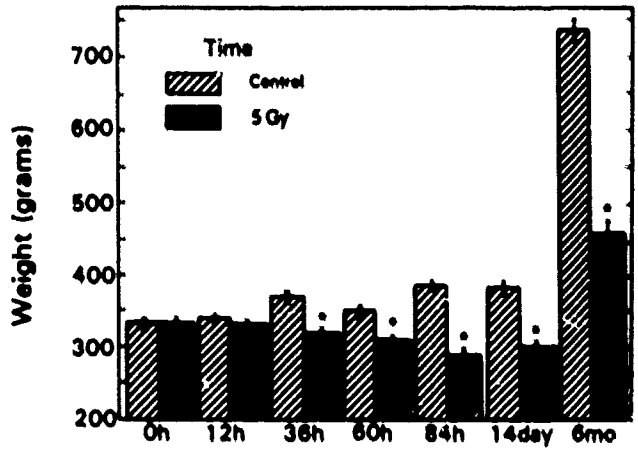

FIG. 1. Body weights at different times after exposure to high-energy iron particles. Values are expressed as grams and represent the means ± SEM. Each group contained 8–10 animals. *The value is significantly different from control at least at the 0.05 level using Student's *t* test.

METHODS

Male Sprague-Dawley Crl:CD(SD)BR rats (Charles River Breeding Laboratories, Kingston, NY) weighing 200–300 g were used in these experiments. Rats were quarantined on arrival and screened for evidence of disease by serology and histopathology before being released from quarantine. The rats were housed individually in polycarbonate isolator cages (Lab Products, Maywood, NJ) on autoclaved hardwood contact bedding ("Beta Chip," Northeastern Products Corp., Warrensburg, NY) and were provided commercial rodent chow ("Wayne Rodent Blok," Continental Grain Co., Chicago, IL) and acidified water (pH 2.5 using HCl) *ad libitum* to minimize *Pseudomonas* infections. Animal holding rooms were kept at 21 ± 1°C with 50 ± 10% relative humidity on a 12-h light:dark lighting cycle with no twilight.

Iron particles were accelerated using the BEVALAC at the Lawrence Berkeley Laboratory (Berkeley, CA) and were delivered to a nominal extraction energy of 600 MeV/amu. This energy made it possible to expose animals in the plateau of the Bragg curve with a residual range in water of 8 cm. Animals were irradiated whole-body unilaterally in well-ventilated restraining tubes (8 × 12 cm) at a rate that averaged 1 Gy/min. The ion beam was Gaussian-shaped with the peak centered on the ion chambers and the heart. The reduction of the dose to the rest of the animal did not exceed 30%. Dosimetry was performed as described previously (*11*). The dose-measuring ionization chamber was located at the center of the beam near the animal.

The rats were irradiated with high-energy electrons delivered from a linear accelerator at the Armed Forces Radiobiology Research Institute (AFRRI). The animals were irradiated unilaterally in well-ventilated polyethylene restraining tubes with electrons accelerated to an energy of 18.1 MeV at 0.44 A. Pulses of about 0.13 Gy each were delivered at a rate of 15/s, each with a duration of 4 μs. All doses were midline tissue doses. Dosimetry was performed using 0.05-cm^3 tissue-equivalent ion chambers that were calibrated by the National Bureau of Standards. Measurements indicated that the electron beam was uniform to within 10%.

Gamma photon irradiation was accomplished using a ^{60}Co source at the AFRRI. The animals were exposed bilaterally to γ photons at a rate of 20 Gy/min. Dosimetry was performed using paired 50-ml ion chambers. Delivered dose was expressed as a ratio of the dose measured in a tissue-equivalent plastic phantom enclosing a restraining tube to that measured in air.

At various times after radiation exposure, the animals were weighed then decapitated, and the brains were excised. The caudate nuclei were obtained by blunt dissection on an ice-cold metal plate. A midsaggital cut was made through the brains and each lateral ventricle was opened to locate the striated caudate nuclei. They were easily scraped out using as the lateral boundary the white matter separating the nuclei from the cerebral cortex. Tissue samples were immediately frozen on dry ice for about 2 h. To minimize day-to-day variation, tissue from sham-irradiated controls was obtained in the same manner each day as tissue from irradiated animals.

The frozen tissue samples were homogenized in 40 vol of ice-cold, 0.4 *N* perchloric acid containing 0.05% sodium metabisulfite and 0.1% EDTA. All the samples were centrifuged at 40,000*g* for 20 min and the supernatants filtered through 0.2-μm Metricel membrane filters. The samples were then stored at −80°C.

Each sample was analyzed within 30 days for the concentrations of dopamine and its metabolites using automated high-performance liquid chromatography as described previously (*12*). The HPLC consisted of a Varian Model 5000 ternary chromatograph, a Varian Vista 401 Data system, a Varian Model 8055 Autosampler, and an air-actuated injector with a 50-μl loop.

On the day of analysis, the samples were thawed and 50 μl of the filtrate without further purification was injected onto a Waters, 10-μm particle, μBondapak C_{18} reverse-phase column (30 × 0.39 cm). The mobile phase consisted of 4 m*M* 1-heptanesulfonic acid, 100 m*M* EDTA, and 1% (v/v) acetonitrile, buffered to pH 3.5. Five minutes after the injection of the sample, a second mobile phase containing 20% acetonitrile instead of 1% was added as a linear gradient over the next 25 min. The peaks were detected with a Bioanalytical Systems L-4 amperometric detector using a glassy carbon electrode. The detector potential was set at 0.72 V versus a Ag/AgCl reference electrode with a sensitivity of 20 nA/V. The areas under the peaks were compared to those of standards using the computer in the Data system. Concentrations were expressed as picomoles per milligram of protein. Protein concentrations in the original homogenates were determined by the method of Lowry *et al.* (*13*).

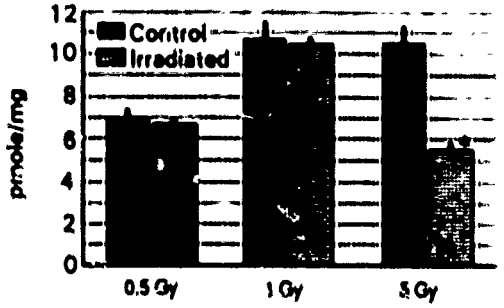

FIG. 2. Concentrations of 3-MT in the caudate nucleus after a single dose of high-energy iron particles. Values are expressed as picomoles per milligram of protein and represent the means ± SEM. Each group contained 8–10 animals. *The value is significantly different from control at least at the 0.05 level using Student's *t* test.

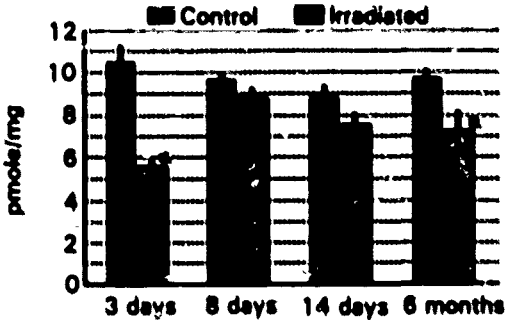

FIG. 3. Concentrations of 3-MT in the caudate nucleus at different times after a 5-Gy dose of high-energy iron particles. Values are expressed as picomoles per milligram of protein and represent the means ± SEM. Each group contained 8–10 animals. *The value is significantly different from control at least at the 0.05 level using Student's *t* test.

RESULTS

Exposure to high-energy iron particles had little effect on the general behavior of the animals. However, after the highest dose (5 Gy) the animals did not gain weight at the same rate as controls. The weights of the irradiated animals were significantly less than those of controls 36 h to 6 months after irradiation (Fig. 1). No effect on body weight was observed after exposure to 0.5 or 1 Gy (data not shown).

Dopamine metabolism in the caudate nucleus was significantly altered after exposure to high-energy iron particles. Three days after irradiation, a dose of 5 Gy reduced 3-MT concentrations by 47% (Fig. 2). DOPAC and HVA concentrations were also reduced but to a lesser extent (Table I). No change in dopamine concentrations was observed. Doses of 0.5 and 1 Gy were ineffective. Also, no effect was observed in any of the substances measured at earlier times (data not shown). The reduction in 3-MT concentrations was not long-lived and disappeared by 8 and 14 days after irradiation (Fig. 3). However, by 6 months after irradiation 3-MT concentrations were again less than control values.

Since previous results from our laboratory suggest that iron particles are significantly more effective than high-energy electrons or γ photons in inducing behavioral and neural changes in rats (*6, 7*), experiments were undertaken to determine if equivalent alterations in striatal 3-MT concentrations could be observed 3 days after exposure to the two low-LET radiations. The results of these experiments indicated that no significant differences occurred in the concentrations of 3-MT after exposure to 5 Gy of either high-energy electrons or γ photons (Fig. 4).

To determine whether higher doses of low-LET radiation could alter 3-MT concentrations, rats were exposed to high-energy electrons in doses of 15, 50, and 100 Gy. Thirty minutes after exposure, the concentrations of 3-MT were significantly reduced in the caudate nucleus (Fig. 5). Similar results were found for DOPAC and HVA concentrations (Table II). Again, no effect on dopamine concentrations were observed. The reduction in metabolite concentrations were found only after 50 or 100 Gy of radiation and was

TABLE I

DOPAC, HVA, and Dopamine Concentrations in the Caudate Nucleus 3 Days after a 5-Gy Dose of High-Energy Iron Particles

	DOPAC	HVA	Dopamine
Control	67.1 ± 5.0	43.4 ± 2.5	536 ± 11.1
Irradiated	50.6 ± 3.1*	31.3 ± 1.8*	540 ± 25.4

Note Each value represents the mean ± SEM of 10 animals.

* The value is significantly different from control at least at the 0.05 level using Student's *t* test.

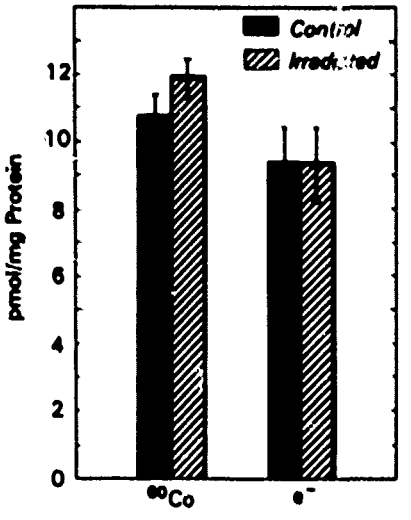

FIG. 4. Concentrations of 3-MT in the caudate nucleus after a 5-Gy dose of γ photons or high-energy electrons. Values are expressed as picomoles per milligram of protein and represent the means ± SEM. Each group contained 9–12 animals.

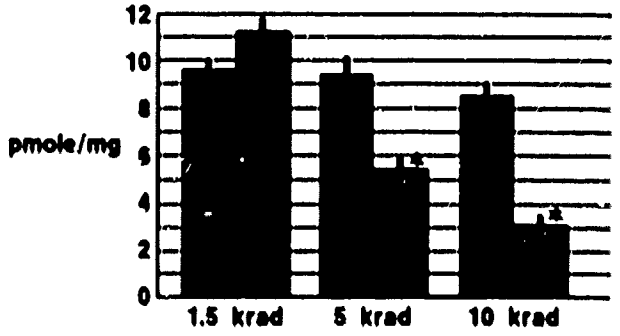

FIG. 5. Concentrations of 3-MT in the caudate nucleus 30 min after a single dose of high-energy electrons. Values are expressed as picomoles per milligram of protein and represent the means ± SEM. Each group contained 6–10 animals. *The value is significantly different from control at least at the 0.05 level using Student's *t* test.

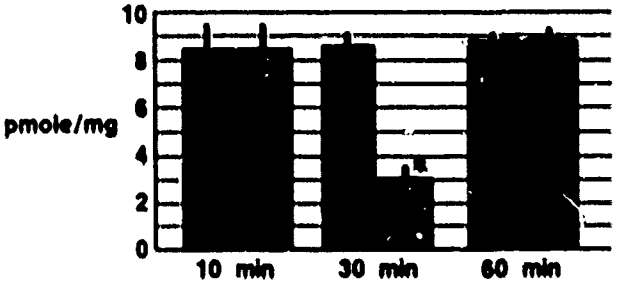

FIG. 6. Concentrations of 3-MT in the caudate nucleus at different times after a 100-Gy dose of high-energy electrons. Values are expressed as picomoles per milligram of protein and represent the means ± SEM. Each group contained 7–10 animals. *The value is significantly different from control at least at the 0.05 level using Student's *t* test.

transient in that 3-MT concentrations could be observed only at 30 min after irradiation, not at 10 and 60 min (Fig. 6). For comparison, striatal 3-MT concentrations were determined after exposure to γ photons. Even a dose of 200 Gy was ineffective in significantly altering 3-MT concentrations (control, 9.45 ± 0.90; irradiated, 7.61 ± 0.97; $P > 0.05$). (No measurements could be made 3 days after exposure to either low-LET radiation because the animals did not survive.)

DISCUSSION

Ionizing radiation in general has been shown to degrade motor behavior in rats. For example, performance on an accelerating rod has been reported to be significantly impaired during the first 30 min after exposure to high doses of γ photons or high-energy electrons (*14*). In addition, electrons have been found to be more effective than photons in degrading behavior (*14, 15*). Under similar experimental conditions, 3-MT concentrations were also reduced, requiring at least 50 Gy of high-energy electrons for an effect. Photons induced no significant alterations even after 200 Gy. In addition, exposure to 100 Gy of high-energy electrons increased potassium-stimulated dopamine release *in vitro* (*16*). These results suggested that exposure to ionizing radiation has a deleterious effect on motor performance and that this effect may be mediated by dopaminergic mechanisms in the caudate nucleus.

TABLE II

DOPAC, HVA, and Dopamine Concentrations in the Caudate Nucleus 30 Min After a 100-Gy Dose of High-Energy Electrons

	DOPAC	HVA	Dopamine
Control	69.3 ± 2.0	67.7 ± 2.8	644 ± 29.0
Irradiated	51.8 ± 2.1*	54.7 ± 2.9*	726 ± 34.1

Note Each value represents the mean ± SEM of seven animals.

* The value is significantly different from control at least at the 0.05 level using Student's *t* test.

The data presented here support the hypothesis that exposure to heavy particles also significantly alters striatal dopaminergic mechanisms but does so at much lower doses than those required for low-LET radiations. In previous experiments, exposure to 0.1–5 Gy of high-energy iron particles disrupted a motor task and significantly depressed striatal oxotremorine-enhanced, potassium-stimulated dopamine release *in vitro*, an effect that lasted at least 14 days (*7*). The results of the present study indicate that doses of 5 Gy of high-energy iron particles induced a short-lived reduction 3 days after exposure in the concentrations of 3-MT (an index of dopamine release (*9, 10*)) as well as those of DOPAC and HVA in the caudate nucleus. This observation supports the idea that the exposure to radiation lowered the turnover and release of dopamine. However, since the activity of the enzymes responsible for the metabolism of dopamine was not measured in this study, it cannot be ruled out that the effects observed are not due to an action of radiation on these enzymes. The effect of radiation on 3-MT concentrations appears to be specific to the caudate nucleus, since equivalent responses were not observed in other brain regions (data not shown).

It has been demonstrated previously that the synthesis and turnover of dopamine and the concentrations of dopamine metabolites in the nigrostriatal pathways depend on the level of impulse flow through these fibers. When dopaminergic fibers are electrically stimulated or when drugs that stimulate impulse flow through these fibers are administered, dopamine synthesis and the concentrations of dopamine metabolites are elevated (*8, 9*). On the other hand, when impulse flow is severely decreased by treating animals with γ-butyrolactone or by placing lesions in the nigrostriatal pathways, the rate of synthesis and concentration of dopamine are elevated (*17*), while the concentrations of the dopamine metabolites are reduced (*10, 18, 19*). Both of

these effects are prevented by pretreatment with dopaminergic agonists that stimulate presynaptic dopaminergic receptors and are augmented by dopaminergic antagonists (*20–22*). Exposure to doses of high- or low-LET radiation seems to have effects on dopamine metabolism similar to those of γ-butyrolactone treatment or nigrostriatal lesions on striatal dopaminergic pathways.

There is other evidence using a variety of approaches and exposure to low-LET radiation that is consistent with the hypothesis that ionizing radiation can induce a specific reduction in the activity of nigrostriatal dopaminergic pathways. Since these pathways exert a tonic inhibitory influence on cholinergic fibers within the caudate nucleus (*23–25*), a reduction in this inhibitory input would result in an elevated release of acetylcholine. Exposure to ionizing radiation has been shown to increase *in vitro* striatal high-affinity choline uptake (*16*), an index of acetylcholine release (*23*), that is transient and is observed only during the time course of the alterations in dopaminergic activity (*16*).

Another approach involves electrical stimulation of the substantia nigra and lateral hypothalamus, which normally increases locomotor activity. Thirty minutes after irradiation with 100 Gy of high-energy electrons, nigral stimulation is less effective than lateral hypothalamic stimulation in increasing activity (*26*), suggesting that the nigrostriatal fibers are hypoactive. In further support, administration of amphetamine or apomorphine, two dopaminergic agonists, can antagonize radiation-induced behavioral depression, but the dose–response curves of these agonists are shifted to the right, indicating a reduced sensitivity to the drugs (unpublished observations). Conversely, animals exposed to ionizing radiation are more sensitive to dopaminergic antagonists (*27*). Radiation exposure enhances the cataleptogenic effect of haloperidol and the ability of the drug to stimulate potassium-stimulated dopamine release *in vitro*.

In conclusion, the data presented provide further evidence that exposure to ionizing radiation alters dopaminergic transmission. In addition, the effectiveness of high-energy iron particles appears to be at least 10 times that observed for low-LET radiation. Deficits in motor function may occur as a result of these alterations in dopaminergic transmission and may be of concern for long-term travelers in space. Whether the mechanisms underlying the effects after exposure to high- or low-LET radiation are the same, given the differences in time course and sensitivity, will await the results of further research.

ACKNOWLEDGMENTS

The authors acknowledge the assistance of Drs. E. John Ainsworth, Patricia Durbin, and Bernhard Ludewigt and the staff at the Lawrence Berkeley Laboratory without whose help these studies could not have been undertaken. This research was supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under Work Unit B4157. The views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred. Research was conducted according to the principles enunciated in the *Guide for the Care and Use of Laboratory Animal Resources*, National Research Council.

RECEIVED: December 12, 1988; ACCEPTED: September 12, 1989

REFERENCES

1. D. M. RUST, Solar flares, proton showers, and the space shuttle. *Science* **216**, 939–946 (1982).
2. E. G. STASSINOPOULOS, The earth's trapped and transient space radiation environment. In *Terrestrial Space Radiation and Its Biological Effects* (P. D. McCormack, C. E. Swenberg, and H. Bücker, Eds.), pp. 5–35. Plenum, New York, 1988.
3. S. CURTIS and M. C. WILKINSON, *Study of Radiation Hazards to Man on Extended Mission*. Report Cr-1037, NASA, Washington, DC, 1968.
4. A. L. RILEY and D. L. TUCK, Conditioned taste aversions: A behavioral index of toxicity. *Ann. N.Y. Acad. Sci.* **443**, 272–292 (1985).
5. L. VALZELLI, *Psychopharmacology: An Introduction to Experimental and Clinical Principles*. Spectrum, New York, 1973.
6. B. M. RABIN, W. A. HUNT, and J. A. JOSEPH, An assessment of the behavioral toxicity of high-energy iron particles compared to other qualities of radiation. *Radiat. Res.* **119**, 113–122 (1989).
7. J. A. JOSEPH, W. A. HUNT, B. M. RABIN, and T. K. DALTON, Correlative motor behavioral and striatal dopaminergic alterations induced by ^{56}Fe radiation. In *Terrestrial Space Radiation and Its Biological Effects* (P. D. McCormack, C. E. Swenberg, and H. Bücker, Eds.), pp. 553–571. Plenum, New York, 1988.
8. D. F. SHARMAN, The catabolism of catecholamines. *Br. Med. Bull.* **29**, 110–115 (1973).
9. W. KEHR, 3-Methoxytyramine and normetanephrine as indicators of dopamine and noradrenaline release in mouse brain *in vivo*. *J. Neural Transm.* **50**, 165–178 (1981).
10. P. L. WOOD, H. S. KIM, and M. R. MARIEN, Intracerebral dialysis: Direct evidence for the utility of 3-MT measurements as an index of dopamine release. *Life Sci.* **41**, 1–5 (1987).
11. E. A. BLAKELY, C. A. TOBIAS, T. C-H. YANG, K. C. SMITH, and J. T. LYMAN, Inactivation of human kidney cells by high-energy, monoenergetic heavy ion beams. *Radiat. Res.* **80**, 122–160 (1979).
12. W. A. HUNT and T. K. DALTON, An automated method for the determination of biogenic amines and their metabolites by high performance liquid chromatography. *Anal. Biochem.* **135**, 269–274 (1988).
13. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL, Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275 (1951).
14. V. BOGO, Effects of bremsstrahlung and electron radiation on rat motor performance. *Radiat. Res.* **100**, 313–320 (1984).
15. W. A. HUNT, Comparative effects of exposure to high-energy electrons and gamma radiation on active avoidance behavior. *Int. J. Radiat. Biol.* **44**, 257–260 (1983).
16. W. A. HUNT, T. K. DALTON, and J. H. DARDEN, Transient alterations in neurotransmitter activity in the caudate nucleus of rat brain after a high dose of ionizing radiation. *Radiat. Res.* **80**, 556–562 (1979).
17. J. R. WALTERS, R. H. ROTH, and A. G. AGHAJANIAN, Dopaminergic neurons: Similar biochemical and histochemical effects of *gamma*-hydroxybutyrate and acute lesions of the nigro-neostriatal pathway. *J. Pharmacol. Exp. Ther.* **186**, 630–639 (1973).

18. P. C. WALDMEIER, J. LAUBER, W. BLUM, and W. J. RICHTER, 3-Methoxytyramine: Its suitability as an indicator of synaptic dopamine release. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 315, 219–225 (1981).

19. R. H. ROTH, I. C. MURRIS, and J. R. WALTERS, Central dopaminergic neurons: Effects of alterations in impulse flow on the accumulation of dihydroxyphenylacetic acid. *Eur. J. Pharmacol.* 36, 163–171 (1976).

20. J. R. WALTERS and R. H. ROTH, Dopaminergic neurons: Drug-induced antagonism of the increase in tyrosine hydroxylase activity produced by cessation of impulse flow. *J. Pharmacol. Exp. Ther.* 191, 82–91 (1974).

21. J. R. WALTERS and R. H. ROTH, Dopaminergic neurons—Alteration in the sensitivity of tyrosine hydroxylase to inhibition by endogenous dopamine after cessation of impulse flow. *Biochem. Pharmacol.* 25, 649–654 (1976).

22. S. M. WUERTHELE and K. F. MOORE, Effects of dopaminergic antagonists on striatal DOPAC concentrations and *alpha*-methyl-*para*-tyrosine-induced decline of dopamine following intrastriatal injections of kainic acid. *J. Pharm. Pharmacol.* 31, 180–182 (1979).

23. S. J. ATWEH, R. SIMON, and M. J. KUHAR, Utilization of sodium-dependent, high affinity choline uptake *in vitro* as a measure of the activity of cholinergic neurons *in vivo*. *Life Sci.* 17, 1535–1544 (1975).

24. S. CONSOLO, H. LADINSKY, and S. GARATTINI, Effect of several dopaminergic drugs and trihexaphenidyl on cholinergic parameters in the rat striatum. *J. Pharm. Pharmacol.* 26, 275–277 (1974).

25. H. STADLER, K. G. LLOYD, M. GADEA-CIRIA, and G. BARTHOLINI, Enhanced striatal acetylcholine release by chlorpromazine and its reversal by apomorphine. *Brain Res.* 55, 476–480 (1973).

26. G. A. MICKLEY and H. TEITELBAUM, Persistence of lateral hypothalamic-mediated behaviors after a supralethal dose of ionizing radiation. *Aviat. Space Environ. Med.* 49, 868–873 (1978).

27. J. A. JOSEPH, S. B. KANDASAMY, W. A. HUNT, T. K. DALTON, and S. STEVENS, Radiation-induced increases in sensitivity of cataleptic behavior to haloperidol: Possible involvement of prostaglandins. *Pharmacol. Biochem. Behav.* 29, 335–341 (1988).

Adv. Space Res. Vol. 9, No. 10, pp. (10)333–(10)336, 1989
Printed in Great Britain

0273-1177/89 $0.00 + .50
1989 COSPAR

BEHAVIORAL AND NEUROCHEMICAL ABNORMALITIES AFTER EXPOSURE TO LOW DOSES OF HIGH-ENERGY IRON PARTICLES

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ABSTRACT

Exposure of rats to high-energy iron particles (600 MeV/amu) has been found to alter behavior after doses as low as 10 rads. The performance of a task that measures upper body strength was significantly degraded after irradiation. In addition, an impairment in the regulation of dopamine release in the caudate nucleus (a motor center in the brain), lasting at least 6 months, was also found and correlated with the performance deficits. A general indication of behavioral toxicity and an index of nausea and emesis, the conditioned taste aversion, was also evident. The sensitivity to iron particles was 10-600 times greater than to gamma photons. These results suggest that behavioral and neurobiological damage may be a consequence of exposure to low doses of heavy particles and that this possibility should be extensively studied.

INTRODUCTION

The prospect of long-term space travel beyond Earth orbit raises the question about the safety of astronauts. Among potential hazards in space, radiation exposure is an important one to consider in setting safety standards. Although research has considered the carcinogenic and genetic consequences of long-term exposure to radiation, little effort has been expended to determine whether there may be acute adverse effects of exposure, such as might be found during a solar particle event.

Solar particle events are composed mostly of high-energy protons. During major solar events, the flux of energetic heavy particles can rapidly increase several orders of magnitude above the galactic background. Also, heavy particles constitute about 1% of the galactic cosmic radiation with energies ranging to an excess of 10 GeV/nucleon. The importance of heavy particles in spite of their small abundance compared to protons is the problem of shielding. Consequently, the possible deleterious effects of exposure to these particles cannot be ignored.

The efficient performance of assigned duties in a spacecraft is important for the successful completion of a mission and under some circumstances possibly for the survival of astronauts during periods of emergency. Thus, knowledge of behavioral capabilities and possible alterations in brain function must be acquired in order to properly assess this question. An effort has been made in this laboratory to examine the effects of high-energy iron particles on behavior and brain function. The endpoints measured included a general measure of behavioral toxicity (the conditioned taste aversion), which is also an index of nausea and emesis, several motor tasks, and neurochemical endpoints that measure the release and turnover of dopamine from slices of the caudate nucleus. Irradiations of male, Sprague-Dawley rats were performed at the Bevalac of the Lawrence Berkeley Laboratory using the plateau region of an iron-56 beam with an energy of 600 MeV/amu.

CONDITIONED TASTE AVERSION LEARNING

Over the course of evolution animals have developed mechanisms that help prevent accidental poisoning, the best-known one being the emetic response. Emesis can occur as a result of consuming presumably tainted food that is then expelled from the stomach. In addition to emesis, animals are also capable of avoiding potentially toxic substances after a single ingestion of quantities less toxic than those required to induce vomiting. This involves a process called the conditioned taste aversion (CTA). A CTA develops when the animal associates the taste of novel tasting food with a physiological response, possibly related to illness, and then subsequently avoids ingestion of that food. In a laboratory setting, a CTA is typically induced by pairing a normally preferred but novel tasting fluid with exposure to a toxin. The animal will then avoid drinking the fluid when presented again.

The CTA paradigm in the rat can be used as a general measure of behavioral toxicity and as a model to study the mechanisms by which exposure to non-lethal levels of ionizing radiation can

produce changes in behavior. Because the functional effects of emesis and taste aversion learning are similar, in the sense that they limit the intake and/or absorption of toxic substances, it can be argued that the CTA paradigm also represents a model for the study of radiation-induced nausea and emesis. Therefore, the CTA can provide an index of the probability that nausea and emesis will occur.

The CTA induced by ionizing radiation has been extensively studied and a clearer idea of how it develops has been emerging /1/. The most important discovery is the involvement of a specific nucleus in the brain stem, the area postrema. The area postrema has been demonstrated to play a critical role in the development of CTAs induced by a broad range of unrelated toxins, and lesions placed in the area postrema block these toxin-induced CTAs.

Doses of 20-50 rads of high-energy iron particles induced a CTA, the intensity of which was dose-dependent /2/3/. Exposure to gamma photons, high-energy electrons, or fission neutrons also induced a CTA, but larger doses than with the heavy ion were required. A maximal CTA was observed only after exposure to 500 rads of photons or electrons, compared to only 50 rads for high-energy iron particles, suggesting that iron particles are 10 times more effective than gamma photons. The potency of neutrons was between that of iron particles and the low-LET radiations. In addition, similar to gamma photon irradiation, lesions in the area postrema could block the CTA induced by iron particles.

MOTOR BEHAVIOR

Previous studies have indicated that motor performance is degraded in a dose-dependent manner after exposure to ionizing radiation, when animals were placed on tasks for which physical strength, endurance, and coordination were required (see review of Hunt /4/). These studies have generally utilized radiation sources ranging from mixed neutron-gamma radiation to high-energy electrons. The effect of exposure to heavy charged particles on motor behavior has not been studied. In an attempt to ascertain whether high-energy iron particles will degrade motor behavior, animals were tested on a battery of four motor tests as described previously /5/.

Exposure to iron particles induced deficits only on the wire suspension task /5/. Irradiated animals remained suspended on the wire for significantly shorter times than controls after exposure to all doses of radiation from 10 to 500 rads. There were, however, quantitative differences in performance among the various irradiated groups depending on the dose and time after exposure. For example, 3 days after irradiation, the animals that received 500 rads did not show any deficits in performance on this task. However, at 8 and 14 days, performance on the task was significantly depressed after all doses.

NEUROCHEMICAL STUDIES

The precise locus of the behavioral deficits observed after irradiation is not known. There is, however, a great deal of evidence in non-irradiated animals to suggest that the deficits in motor behavior may be mediated by the nigrostriatal system, one of the basic central processing areas involved in the mediation of motor behavior. This system appears to control a wide variety of motor responses ranging from the simple (balance and coordination) to the complex (the ordering and sequencing of intricate behavioral patterns directed by external stimuli). Since the control of such complicated patterns of behavior depends upon the coordination of a host of neurotransmitters and neuromodulators, disruptions of the interactions among them, as a result of heavy particle bombardment in a space environment, may result in decrements in motor function.

Of the many intrastriatal neurotransmitter systems involved in mediating these types of behaviors, two of the more important ones involve the neurotransmitters dopamine and acetylcholine. These transmitters exert part of their control through reciprocal inhibition. Reciprocal inhibition is a regulatory mechanism that modulates the release of dopamine. In the striatum, this is accomplished through autoreceptors on dopaminergic terminals, which when stimulated, decrease the firing rate of the neuron. If these autoreceptors are stimulated by dopamine agonists or inhibited by dopamine antagonists, dopamine release will be inhibited or enhanced, respectively. The autoreceptors are in turn controlled by a group of inhibitory cholinergic receptors of the muscarinic type. Muscarinic agonists activate these receptors, which then inhibit the dopamine autoreceptors, and potentiate dopamine release from the striatum.

A study of possible radiation-induced reductions of autoreceptor function related to the deficits in motor behavior was undertaken with animals irradiated with iron particles /5/6/. Analysis of K^+-evoked release of dopamine from striatal slices in vitro indicated irradiation with doses of 50-500 rads significantly increased release 3 days later. (These results were similar to those obtained shortly after irradiation with 10,000 rads of high-energy electrons /7/). However, oxotremorine enhancement of K^+-evoked release of dopamine was reduced after exposure to 10-100 rads. (Preliminary results indicate that at least 6000 rads of high-energy electrons are needed to have an effect.) Paradoxically, no reduction of oxotremorine enhancement of

K^+-evoked release of dopamine was observed 3 days after irradiation with 500 rads. Deficits did not appear until 8 and 14 days after irradiation. Six months after irradiation, little recovery in the deficits could be observed.

Interestingly, the pattern of alterations in oxotremorine-enhanced dopamine release paralleled the deficits observed on the wire suspension task after all doses of radiation studied. In fact, the 500-rad group showed neither the behavioral deficits nor the reduction in dopamine release 3 days after irradiation, while at 8 and 14 days, deficits were observed in both parameters. In addition, iron particles were at least 600 times more effective than gamma photons. These effects of high-energy iron particles are similar to those found in ageing animals /8/, suggesting that irradiation may accelerate the ageing process.

Since a 500-rad dose of iron particles had no effect on the wire suspension task nor on muscarinic control of dopamine release 3 days after exposure, other factors may have played a role in the response obtained. In support of this possibility, there were general behavioral and physiological abnormalities noted at this time period. The animals displayed reduced muscle tone, hind-limb tremors, and hypothermia /2/. However, by 14 days after exposure, these signs had disappeared. In addition, the animals did not gain weight at the same rate as controls /9/. The weights of the irradiated animals were significantly less than those of controls 14 days and 6 months after irradiation. No effect on body weight was observed after exposure to 50 or 100 rads.

To assess other possible abnormalities in dopaminergic activity, the metabolism of dopamine in vivo was studied. The concentrations of dopamine metabolites have been used as an index of the rate of utilization (turnover) of dopamine by neurons that use it as a neurotransmitter. The concentration of 3-methoxytyramine (3-MT), an O-methylated metabolite of dopamine, in particular, has been shown to correlate best with dopamine release. In the present experiments, 3-MT concentrations were determined after various doses of high-energy iron particles and at different times after irradiation.

Dopamine metabolism in the caudate nucleus was significantly altered after exposure to high-energy iron particles. Three days after irradiation, a dose of 500 rads reduced 3-MT concentrations by 47% /9/. Doses of 50 and 100 rads were ineffective. This effect was not long-lived and disappeared by 8 and 14 days after irradiation. However, by 6 months after irradiation 3-MT concentrations were again decreased.

Since previous results from our laboratory as discussed above suggest that iron particles are significantly more effective than gamma photons or high-energy electrons in inducing behavioral and neural changes, experiments were undertaken to determine if equivalent alterations in striatal 3-MT concentrations could be observed 3 days after exposure to the two low-LET radiations. The results of these experiments indicated that no significant differences were observed in the concentrations of 3-MT after exposure to 500 rads of either gamma photons or high-energy electrons.

SUMMARY

Exposure to high-energy iron particles exerts significant effects on behavior and brain function. Measures of general behavioral toxicity and of motor function along with its corresponding neurochemical correlates suggest possible deficits after doses as low as 10 rads. The deficits in motor function may impair the ability of astronauts to perform critical tasks, may not be reversible, and may accelerate the ageing process. More research is required to adequately assess the hazards of acute radiation exposure during space travel beyond the magnetosphere.

ACKNOWLEDGMENTS

The authors wish to acknowledge the assistance of Drs. E. John Ainsworth, Patricia Durbin, and Bernhard Ludewigt and the staff at the Lawrence Berkeley Laboratory without whose help these studies could not have been undertaken. This research was supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under work units B4157. Views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred. Research was conducted according to the principles enunciated in the 'Guide for the Care and Use of Laboratory Animal Resources, National Research Council.'

REFERENCES

1. B.M. Rabin, and W.A. Hunt, Mechanisms of radiation-induced conditioned taste aversion learning. Neurosci. Biobehav. Rev. 10, 55 (1986).

2. W.A. Hunt, B.M. Rabin, J.A. Joseph, T.K. Dalton, W.E. Murray, Jr., and S.A. Stevens, Effects of iron particles on behavior and brain function: Initial studies, in: Terrestrial Space Radiation and its Biological Effects, eds. P.D. McCormack, C.E. Swenberg, and H. Bücker, Plenum Press, New York 1988, in press.

3. B.M. Rabin, W.A. Hunt, and J.A. Joseph. An assessment of the behavioral toxicity of high-energy iron particles compared to other radiations, in preparation (1988).

4. W.A. Hunt, Effects of ionizing radiation on behavior and the brain, in: Military Radiobiology, eds. J.C. Conklin and R.I. Walker, Academic Press, New York 1986, pp. 321-330.

5. J.A. Joseph, W.A. Hunt, B.M. Rabin, and T.K. Dalton, Correlative motor behavioral and striatal dopaminergic alterations induced by Fe-56, in: Terrestrial Space Radiation and its Biological Effects, eds. P.D. McCormack, C.E. Swenberg, and H. Bücker, Plenum Press, New York 1988, in press.

6. J.A. Joseph, W.A. Hunt, and B.M. Rabin, 'Striatal ageing' induced by high-energy iron particles, in preparation (1988).

7. W.A. Hunt, T.K. Dalton, and J.H. Darden, Transient alterations in neurotransmitter activity in the caudate nucleus of rat brain after a high dose of ionizing radiation, Radiat. Res. 80, 550 (1979).

8. J.A. Joseph, T.K. Dalton, and W.A. Hunt, Age-related decrements in the muscarinic enhancement of K^+-evoked release of endogenous striatal dopamine: an indicator of altered cholinergic-dopaminergic reciprocal inhibitory control in senescence. Brain Research 454, 140 (1988).

9. W.A. Hunt, T.K. Dalton, J.A. Joseph, and B.M. Rabin, Reduction in 3-methoxytyramine concentrations in the caudate nucleus after exposure to high-energy iron particles: Evidence for deficits in dopaminergic neurons. In preparation (1988).

RADIATION RESEARCH 121, 84-90 (1990)

Involvement of Prostaglandins and Histamine in Radiation-Induced Temperature Responses in Rats

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KANDASAMY, S. B., AND HUNT, W. A. Involvement of Prostaglandins and Histamine in Radiation-Induced Temperature Responses in Rats. *Radiat. Res.* 121, 84–90 (1990).

Exposure of rats to 1–15 Gy of γ radiation induced hyperthermia, whereas exposure to 20–150 Gy produced hypothermia. Since radiation exposure induced the release of prostaglandins (PGs) and histamine, the role of PGs and histamine in radiation-induced temperature changes was examined. Radiation-induced hyper- and hypothermia were antagonized by pretreatment with indomethacin, a cyclooxygenase inhibitor. Intracerebroventricular administration of PGE2 and PGD2 induced hyper- and hypothermia, respectively. Administration of SC-19220, a specific PGE2 antagonist, attenuated PGE2- and radiation-induced hyperthermia, but it did not antagonize PGD2- or radiation-induced hypothermia. Consistent with an apparent role of histamine in hypothermia, administration of disodium cromoglycate (a mast cell stabilizer), mepyramine (H1-receptor antagonist), or cimetidine (H2-receptor antagonist) attenuated PGD2- and radiation-induced hypothermia. These results suggest that radiation-induced hyperthermia is mediated via PGE2 and that radiation-induced hypothermia is mediated by another PG, possibly PGD2, via histamine. © 1990 Academic Press, Inc

INTRODUCTION

Exposure to ionizing radiation has been reported to induce hyperthermia in cats, rabbits, and humans (*1, 2*), hypothermia in guinea pigs (*3*), a biphasic response in monkeys (a fall followed by a rise) (*4*), and a dual effect in rats (low and high doses produced hyper- and hypothermia, respectively) (*5*). When rats were exposed to low doses of radiation either to the head or to the whole body, hyperthermia was observed, while body-only exposure induced no significant effect (*5*). This suggests that radiation-induced hyperthermia is a result of a direct action on the brain. Radiation-induced hyperthermia can also be attenuated by pre- or post-treatment with indomethacin, a cyclooxygenase inhibitor (*5*). Hyperthermia can also be reduced by the central administration of naloxone, a μ-receptor antagonist, but only after low doses of radiation (*5*). Taken together, these findings suggest that radiation-induced hyperthermia is mediated through the synthesis and release of prostaglandins (PGs) in the brain and to a lesser extent through the release of endogenous opioid peptides.

Radiation induces dramatic increases in the levels of prostaglandins in a variety of tissues (*6–10*) including brain (*11*). The E series of PGs have been identified in the brain as well as in the cerebrospinal fluid and have been suggested as a mediator of hyperthermia by Milton and co-workers (*12–15*), although Cranston *et al.* reported results contradicting this hypothesis (*16*). Several recent studies suggest that rodent brain contains predominantly PGD2 (*17–22*). Mast cells release histamine and also synthesize and subsequently release PGD2 as a major cyclooxygenase metabolite of arachidonic acid after activation by various stimuli (*23*). Histamine and PGD2 induce hypothermia and have been implicated in thermoregulation (*24–27*). In chickens, rabbits, and rats, central administration of histamine has a dual effect. After low doses hypothermia is observed. However, after high doses of histamine, hyperthermia is induced (*28–30*). In sheep, systemic injection of histamine also induced hyperthermia but only at warm ambient temperatures (*31*). Since exposure of rats to radiation-induced hyper- or hypothermia, depending on the dose, and the release of PGs and histamine (*5*), the role of PGs and histamine in radiation-induced temperature changes was investigated using inhibitors/antagonists of PGs and histamine. Also, it was determined whether or not high doses of radiation act on the brain or on peripheral sites.

METHODS

Drugs. The drugs used were PGE2, PGD2, and indomethacin (Sigma Chemical Co., St. Louis, MO); mepyramine maleate (Mallinckrodt Inc., St. Louis, MO); cimetidine (Smith Kline and French Laboratory, Philadelphia, PA); disodium cromoglycate (Fisons Corporation, Bedford, MA); 1-acetyl-2(8-chloro-10,11-dihydro-benz-[b,f]-[1,4]-oxazepine-10-carbonyl)hydrazine (SC-19220) (G. D. Searle Laboratory, Chicago, IL); ketamine hydrochloride (Parke-Davis, Detroit, MI); xylazine (Hayer Lockhart, Shawnee, KS); and acepromazine (Ayerst Laboratories, NY). Mepyramine and disodium cromoglycate were dissolved in sterile, nonpyrogenic saline. Cimetidine was dissolved in 0.1 ml of 1 *N* HCl and diluted to the final volume with saline. PGE2 and PGD2 were stored at −20°C and were dissolved in sterile saline before the injection. SC-19220 was dissolved in a mixture of 40% DMSO and saline.

Animals. Male Sprague–Dawley Crl:CD(SD)BRD rats (Charles River Breeding Laboratories, Kingston, NY) weighing 200–300 g were used in

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TABLE 1
Changes in Rectal Temperature of Rats 15 Min after Exposure to Variable Doses of Ionizing Radiation

Dose (Gy)	Mean Δ temperature (°C)
Sham	+0.1 ± 0.05 (12)
1	+0.2 ± 0.05 (10)
3	+0.4 ± 0.10 (10)
5	+0.5 ± 0.05 (10)*
10	+1.9 ± 0.05 (10)*
15	+0.9 ± 0.10 (10)*
20	0.4 ± 0.05 (10)
50	0.8 ± 0.05 (10)*
100	0.9 ± 0.05 (10)*
150	1.2 ± 0.10 (10)*

* Significantly different from sham irradiation, $P < 0.05$. Values represent the mean ± SE. Numbers in parentheses are the number of animals in each group.

these experiments. Rats were quarantined on arrival and screened for evidence of disease by serology and histopathology before being released from quarantine. The rats were housed individually in polycarbonate isolator cages (Lab Products, Maywood, NJ) on autoclaved hardwood contact bedding ("Beta Chip" Northeastern Products Corp., Warrensburg, NY) and were provided commercial rodent chow ("Wayne Rodent Blok" Continental Grain Co., Chicago, IL) and acidified water (pH 2.5 using HCl) *ad libitum*. Animal holding rooms were kept at 21 ± 1°C with 50 ± 10% relative humidity on a 12-h, light/dark lighting cycle with no twilight.

Radiation exposure. Rats were placed in clear plastic well-ventilated containers for approximately 5 min before irradiation or sham exposure. The animals were then exposed bilaterally to varying doses of γ photons using a ^{60}Co source at a rate of 10 or 20 Gy/min. Lead bricks were used to shield the head (including the neck) or the body (thorax to pelvis). Each animal was placed in a plastic restraining tube that was enclosed in a cave made of lead bricks with a minimum thickness of 10 cm. The bricks were drilled to accept the part of the tube containing either the head or the body of the rat. During the irradiation, the rats were observed with a remote video monitor to verify that the animals did not shift position within the tube. Dosimetry was performed using an exposure-monitoring 50-cc ion chamber. Delivered dose was expressed as a ratio of the dose measured in a tissue-equivalent plastic phantom enclosed in a restraining tube to that measured in air.

Central administration of drugs. Rats were anesthetized with 1 ml/kg of a mixture of ketamine (50 mg/kg), xylazine (5 mg/kg), and acepromazine (1 mg/kg) given intramuscularly and were placed in a rat stereotaxic apparatus (David Kopf Instruments, No. 320). A single cannula was inserted into the lateral ventricle according to coordinates derived from the atlas of Pellegrino *et al.* (32): 0.8 mm posterior to bregma, 2.5 mm lateral. The cannula was lowered until cerebrospinal fluid rose in the cannula. Dental acrylic was used to secure the cannula. After the end of an experiment, injection sites were histologically verified. The volume of injection was always 10 μl. At least 1 week was allowed for recovery before animals were used for experiments. Injections/irradiations were performed at the same time of day (0900) to avoid diurnal variations in temperature. The antagonists (indomethacin, SC-19220, disodium cromoglycate, mepyramine, and cimetidine) were given 30 min before the administration of the radiation/prostaglandins.

Measurement of body temperature. All experiments were performed at an environmental temperature of 22 ± 1°C. The animals were placed in cages 1 h before the beginning of the experiments and body temperature was measured every 15 min over a period of 2 h with thermistor probes inserted approximately 6 cm into the rectum and connected to a datalogger (Minitrend 205). After each experiment, all animals were euthanized immediately with an overdose of carbon dioxide via inhalation.

Statistics. Statistical evaluations were undertaken using analysis of variance with a significance level of $P < 0.05$. Intergroup comparisons were performed using Tukey's test.

RESULTS

Exposure of rats to 1–15 Gy radiation induced hyperthermia, whereas exposure to 20–200 Gy induced hypothermia (Table 1). The onset of these effects was rapid, and they reached their maximum response within 15 min. On the basis of these results, a dose of 10 Gy of radiation was used to study hyperthermia, and a dose of 50 Gy was chosen to study hypothermia.

As can be seen in Fig. 1, hypothermia induced by a 50-Gy dose of γ radiation occurred only after whole-body or head-only exposure, not when the head was shielded. These results are similar to those found after low doses of radiation (5). Since whole-body exposure resulted in the same effect as head-only exposure, subsequent studies used only whole-body exposure to ionizing radiation.

Experiments were undertaken to determine the effect of indomethacin, an inhibitor of prostaglandin synthesis, on radiation-induced changes in body temperature. Pretreatment with 1–5 mg/kg of indomethacin given intraperitoneally inhibited both the hyper- and hypothermia induced by exposure to 10 and 50 Gy of radiation, respectively (Fig. 2). Indomethacin alone had no effect on body temperature.

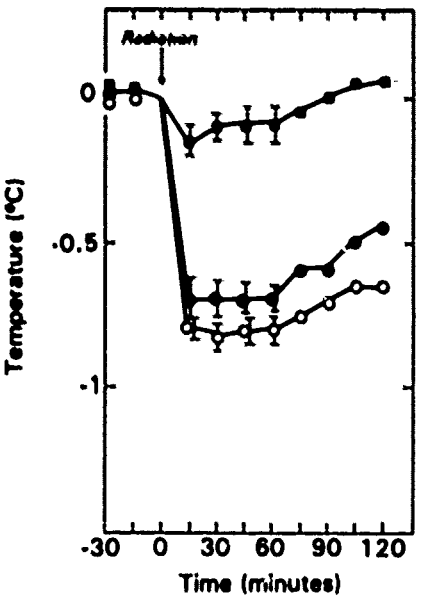

FIG. 1. Effects of 50 Gy of ionizing radiation on body temperature exposed body-only (■), whole-body (●), or head-only (○). Each point represents the mean ± SE of observation of six animals. Zero on the ordinate represents the temperature at the time of irradiation.

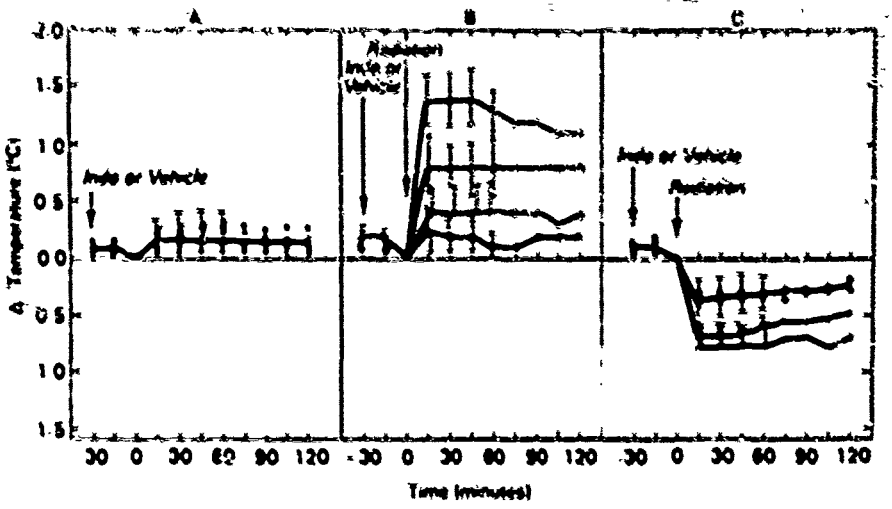

FIG. 2. Effect of indomethacin given ip (Indo) on hyper- and hypothermia induced by 10 and 50 Gy of radiation, respectively. (A) Nonirradiated controls given indomethacin, 1 (○), 3 (△), or 5 mg/kg (□), or vehicle (●); (B) 10 Gy of radiation alone (○) or in the presence of 1 (△), 3 (□), or 5 mg/kg (■) indomethacin; (C) 50 Gy of radiation alone (○) or in the presence of 1 (●), 3 (△), or 5 mg/kg (□) indomethacin. Each point represents the ± SE of observations on five animals. Zero on the ordinate represents the temperature at the time of second injection.

Similar to the effects of low and high doses of radiation, PGE2 (5–20 ng, icv) and PGD2 (10–15 ng, icv) produced dose-dependent hyper- and hypothermia (data not shown). Pretreatment with indomethacin, although it attenuated radiation-induced temperature responses, had no significant inhibitory effect on those of PGE2 or PGD2 (data not shown).

The effect of SC-19220, a PGE2 antagonist, was investigated on PGE2-, PGD2-, and radiation-induced temperature responses. Selected doses of SC-19220 (100–500 ng, icv), which had minimal effects on temperature in control animals, were given prior to PGE2 or PGD2 administration or to exposure to 10 or 50 Gy radiation. The SC-19220 significantly attenuated PGE2- (data not shown) or radiation-induced hyperthermia (Fig. 3) but had no effect on PGD2- (data not shown) or radiation-induced hypothermia (Fig. 3).

Since histamine stored in mast cells throughout the body (*25, 33*) is released by exposure to radiation (*34*), its possible role in thermoregulatory effects of radiation was examined. Disodium cromoglycate is known to be a potent inhibitor of the immunological release of chemical mediators secreted from mast cells (*35*). Disodium cromoglycate (100–500 ng, icv) attenuated PGD2- and radiation-induced hypothermia (Fig. 4).

To examine the role of histaminergic H1 and H2 receptors in PGD2-induced hypothermia, mepyramine (100–300 ng, icv), an H1 antagonist, or cimetidine (100–300 ng, icv), an H2 antagonist, was administered before irradiation. Previous results (*5*) have indicated that mepyramine and cimetidine are specific H1 and H2 receptor antagonists, respectively. Mepyramine antagonized hypothermia induced by 2-methyl histamine, an H1 agonist, but did not antagonize the hypothermia induced by 4-methyl histamine, an H2 agonist. Likewise, cimetidine significantly attenuated the hypothermia induced by 4-methyl histamine but not that induced by 2-methyl histamine. Both mepyramine and cimetidine, which are found to antagonize hypothermia induced by histamine (*5*), attenuated PGD2- and radiation-induced hypothermia (Figs. 5 and 6).

DISCUSSION

As reported earlier, ionizing radiation induced either hyper- or hypothermia in rats depending upon the dose (*5*), changes that appear to be centrally mediated. There are numerous reports that have demonstrated significant increases in PG levels in a variety of tissues including the brain after whole-body irradiation (*6–11*). The observations that PGs induce changes in body temperature and that various anti-inflammatory drugs block the synthesis of PGs in tissue (*24, 36, 37*) have implicated PGs in thermoregulation. Indomethacin attenuates the hyper- or hypothermia caused by low and high doses of ionizing radiation, indicating that this effect may be mediated by PGs. However, it has no antagonistic effect on exogenous administration of PGE2 and PGD2, confirming that it interferes only with PG synthesis. Of the various PGs, it has been reported that only the E series are potent as pyretic agents and that PGD2 is hypothermic (*24, 37*). The present results support these findings. In addition, based on studies using a variety of smooth muscle preparations (*38, 39*) and studies of PGE2-induced fever (*40, 41*), SC-19220, a specific PGE2 antagonist, antagonized only PGE2- and radiation-induced hyperthermia ... duced by low doses of ionizing radiation, suggesting that radiation-induced hyperthermia is mediated by PGE2.

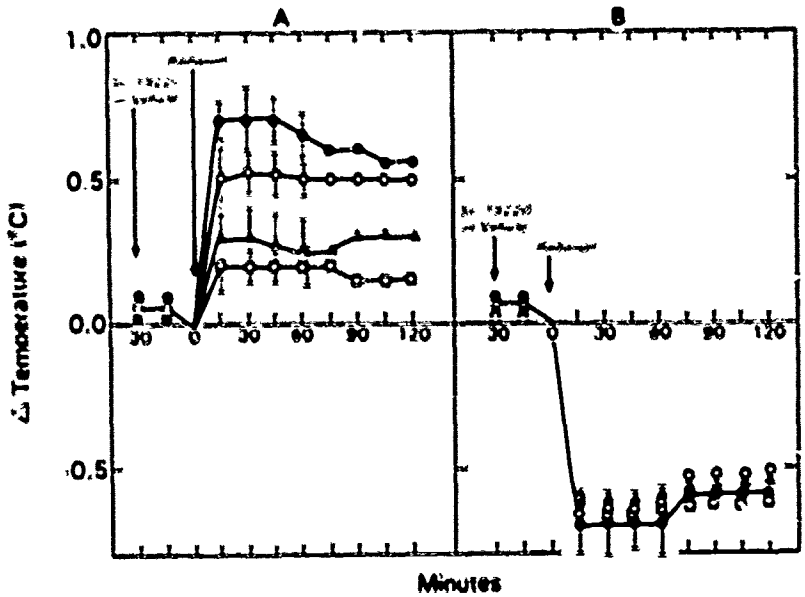

FIG. 3. Effect of SC-19220, icv, on hyper- and hypothermia induced by ionizing radiation. (A) 10 Gy irradiation alone (●) or in the presence of 100 (○), 300 (△), or 500 ng (□) SC-19220. (B) 50 Gy irradiation alone (●) or in the presence of 100 (○), 300 (△), or 500 ng (□) SC-19220. Each point represents the mean ± SE of observations on five animals. Zero on the ordinate represents the temperature at the time of second injection.

Histamine has been implicated in the actions of ionizing radiation including hypotension, reduction in cerebral blood flow, and performance decrements (*42*). Furthermore, concentrations of histamine in circulating blood have been reported to be elevated in humans undergoing radiation therapy (*43*) as well as in dogs and monkeys (*34, 44, 45*) following radiation exposure. Tissue histamine levels are decreased in rats (*46*). Exposure to ionizing radiation resulted in hypothermia which appears to be mediated by the central release of histamine since the hypothermia occurred only after whole-body or head-only exposure, not when the head was shielded.

Histamine is present in high concentrations in the hypothalamus (*47, 48*) and is localized in nerve terminals (*49*), suggesting that it may act as a central neurotransmitter. Also, ascending histamine tracts are found in the median forebrain bundle (*50*); histidine decarboxylase, the enzyme that converts histidine to histamine, is localized in different regions of the brain (*51*); histamine activates adenylate cyclase in the brain (*52*); and brain histamine turnover is increased by stress (*53*). Administration of histidine systemically or histamine centrally evokes hypothermia caused by both H1- and H2-receptor activation (*54*). These neurochemical and pharmacological studies suggest that hista-

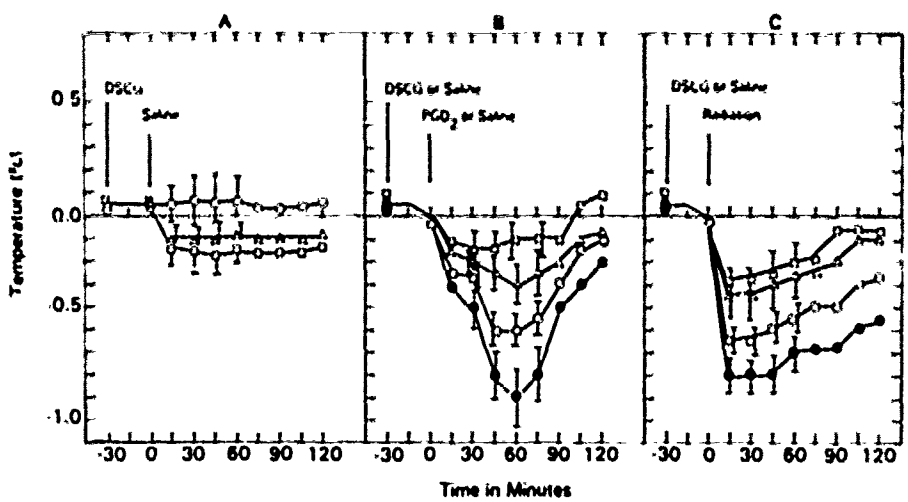

FIG. 4. Effect of disodium cromoglycate (DSCG), icv, on PGD2- and radiation-induced hypothermia. (A) Nonirradiated controls given 100 (○), 300 (△), or 500 ng (□) DSCG; (B) 30 ng of PGD2 alone (●) or in the presence of 100 (○), 300 (△), or 500 ng (□) DSCG; (C) 50 Gy irradiation alone (●) or in the presence of 100 (○), 300 (△), or 500 ng (□) DSCG. Each point represents the mean ± SE of observations of five animals. Zero on the ordinate represents the temperature at the time of second injection.

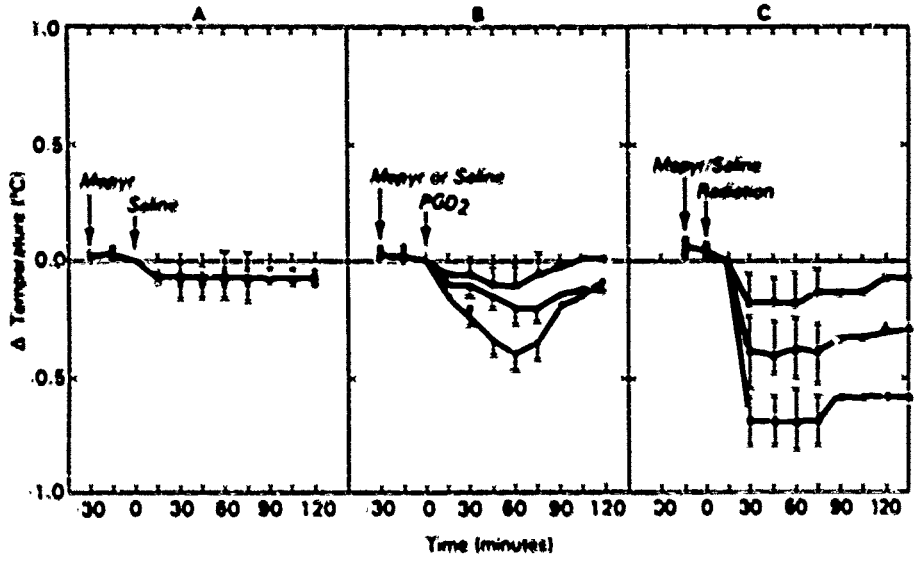

FIG. 5. Effect of mepyramine (Mepyr), icv, on hypothermia induced by PGD2 and ionizing radiation. (A) Nonirradiated controls given 100 (○) or 300 ng (□) mepyramine; (B) 30 ng of PGD2 alone (●) or in the presence of 100 (○) or 300 ng (□) mepyramine. (C) 50 Gy irradiation alone (●) or in the presence of 100 (△) or 300 ng (□) mepyramine. Each point represents the mean ± SE of observations of five animals. Zero on the ordinate represents the temperature at the time of second injection.

mine may be a neurotransmitter involved in many physiological functions including thermoregulation and could underlie radiation-induced hypothermia.

Histamine is stored in mast cells throughout the body (*33*), including the brain, where they are particularly numerous in the hypothalamus (*55, 56*). Arachidonic acid is converted by the cyclooxygenase pathway primarily to PGD2 in mast cells in humans and rats (*17–22*). In addition, mast cells release PGD2 and histamine after activation by various stimuli (*23, 57*). It has been reported that PGD2 potentiates histamine-induced bronchoconstriction in man (*58*) and plasma extravasation in rat skin (*59*). In the present studies, the mast cell stabilizer disodium cromoglycate attenuated radiation- and PGD2-induced hypothermia, suggesting a role of central histamine in this response. The release of histamine acting on both H1 and H2 receptors

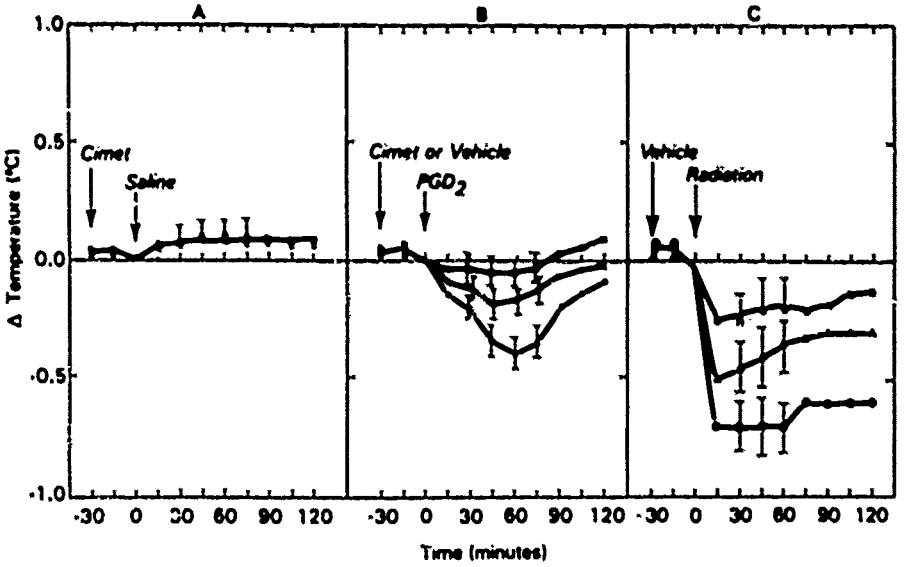

FIG. 6. Effect of cimetidine (Cimet), icv, on hypothermia induced by PGD2 and ionizing radiation. (A) Nonirradiated controls given 100 (○) or 300 ng (□) cimetidine; (B) 30 ng of PGD2 alone (●) or in the presence of 100 (○) or 300 ng (□) cimetidine; (C) 50 Gy irradiation alone (●) or in the presence of 100 ng (△) or 300 ng (□) cimetidine. Each point represents the mean ± SE of observations of five animals. Zero on the ordinate represents the temperature at the time of second injection.

may be involved in radiation-induced hypothermia, since mepyramine, an H1-receptor antagonist, and cimetidine, an H2-antagonist, blocked not only radiation-induced hypothermia but also PGD2-induced hypothermia. Previous results have indicated that serotonin is not involved in radiation-induced hypothermia (5).

In summary, these results suggest that radiation-induced hyperthermia is mediated via PGE2, and histamine is involved in radiation-induced hypothermia. The attenuation of PGD2-induced hypothermia by disodium cromoblycate and antihistamines suggests that PGD2 acts via histaminergic systems.

RECEIVED: May 30, 1989; ACCEPTED: August 24, 1989

REFERENCES

1. T. S. VENISOA, Implications of bioamines in the X-ray temperature response of cats and rabbits, *Radiat. Res.* **48**, 358–367 (1971).
2. H. FANGER and C. C. LUSHBAUGH, Radiation death from cardiovascular shock following a criticality accident, *Arch. Pathol.* **84**, 446–460 (1967).
3. S. B. KANDASAMY and W. HUNT, Involvement of histamine H1 and H2 receptors in hypothermia induced by ionizing radiation in guinea pigs, *Life Sci.* **42**, 555–563 (1988).
4. W. L. MCFARLAND and J. A. WILLIS, *Cerebral Temperature Changes in the Monkey (macaca mulatta) after 2500 rads Ionizing Radiation*, Scientific Report SR4-7, Armed Forces Radiobiology Research Institute, Bethesda, Maryland, 1974.
5. S. B. KANDASAMY, W. HUNT, and G. A. MICKLEY, Implication of prostaglandins and histamine H1 and H2 receptors in radiation-induced temperature responses of rats, *Radiat. Res.* **114**, 42–53 (1988).
6. V. EISEN and D. L. WALKER, Effect of ionizing radiation on a prostaglandin-like activity in tissues, *Br. J. Pharmacol.* **57**, 527–532 (1976).
7. D. P. GIBSON, R. L. DEGOWIN, and S. A. KNAPP, Effect of x-irradiation on release of prostaglandin E from marrow stromal cells in culture, *Radiat. Res.* **89**, 537–545 (1982).
8. L. K. STEEL and G. N. CATRAVAS, Radiation-induced changes in production of prostaglandins F2a, E, and thromboxane B2 in guinea pig parenchymal lung tissues, *Int. J. Radiat. Biol.* **42**, 517–530 (1982).
9. L. K. STEEL, I. K. SWEEDLER, and G. N. CATRAVAS, Effects of ^{60}Co radiation on synthesis of prostaglandins F2a, E, and thromboxane B2 in lung airways of guinea pigs, *Radiat. Res.* **94**, 156–165 (1983).
10. P. J. TROCHA and G. N. CATRAVAS, Prostaglandin levels and lysosomal enzyme activities in irradiated rats, *Int. J. Radiat. Biol.* **38**, 503–515 (1980).
11. E. PAUSESCU, R. CHIRVASIE, T. TEODOSIU, and C. PAUN, Effects of ^{60}Co gamma-radiation on the hepatic and cerebral levels of some prostaglandins, *Radiat. Res.* **65**, 163–171 (1976).
12. W. FELDBERG, K. P. GUPTA, A. S. MILTON, and S. WENDLANDT, Effect of pyrogen and antipyretic on prostaglandin activity in cisternal C.S.F. of unanaesthetized cats, *J. Physiol.* **234**, 279–303 (1973).
13. A. S. MILTON, S. SMITH, and K. B. TOMKINS, Levels of prostaglandin F and E in cerebrospinal fluid of cats during pyrogen-induced fever, *Br. J. Pharmacol.* **59**, 447–448 (1977).
14. W. K. PHILLIP-DORMSTON and R. SIEGERT, Prostaglandins of the E and F series in rabbit cerebrospinal fluid during fever induced by Newcastle disease virus, E. coli-endotoxin, or endogenous pyrogen, *Med. Microbiol. Immunol.* **159**, 279–284 (1974).
15. C. A. HARVEY, A. S. MILTON, and D. W. STRAUGHAN, Prostaglandin E levels in cerebrospinal fluid of rabbits and the effects of bacterial pyrogen and antipyretic drugs, *J. Physiol., Lond.* **248**, 26–27P (1975).
16. W. I. CRANSTON, R. F. HELLON, and D. MITCHELL, A dissociation between fever and prostaglandin concentration in cerebrospinal fluid, *J. Physiol., Lond.* **253**, 583–592 (1975).
17. M. S. ABDEL-HALIM, M. HAMBERG, B. SJOQUIST, and E. ANGGARD, Identification of prostaglandin D2 as the major prostaglandin in homogenates of rat brain, *Prostaglandins* **14**, 633–643 (1977).
18. M. S. ABDEL-HALIM and E. ANGGARD, Regional and species differences in endogenous prostaglandin biosynthesis by brain homogenates, *Prostaglandins* **17**, 411–418 (1979).
19. F. F. SUN, J. P. CHAPMAN, and J. C. MCGUIRE, Metabolism of prostaglandin endoperoxide in animal tissues, *Prostaglandins* **14**, 1055–1074 (1977).
20. R. J. GAUDET, I. ALAM, and L. LEVINE, Accumulation of cyclooxygenase products of arachidonic acid metabolism in gerbil brain during reperfusion after bilateral common carotid artery occlusion, *J. Neurochem.* **35**, 653–658 (1980).
21. E. BERCHTOLD-KANZ, H. ANHUT, R. HELDT, B. B. NEUFANG, and G. HERTTING, Regional distribution of arachidonic acid metabolites in rat brain following convulsive stimuli, *Prostaglandins* **22**, 67–79 (1981).
22. U. FORSTERMANN, R. HELDT, F. KNAPPEN, and G. HERTTING, Potential anti-convulsive properties of endogenous prostaglandins formed in mouse brain, *Brain Res.* **248**, 303–310 (1982).
23. C. W. PARKER, In *Fundamental Immunology* (W. E. Paul, Ed.), pp. 709–747, Raven Press, New York, 1984.
24. W. G. CLARK and J. M. LIPTON, Changes in body temperature after administration of acetylcholine, histamine, morphine, prostaglandins and related agents: II, *Neurosci. Biobehav. Rev.* **9**, 479–552 (1985).
25. J. C. SCHWARTZ, J. M. ARRANG, and M. GARBARG, Three classes of histamine receptors in brain, *TIPS* **7**, 24–28 (1986).
26. J. C. SCHWARTZ, G. BARBIN, A. M. DUCHEMIN, M. GARBARG, C. LLORENS, H. POLLARD, T. T. QUACH, and C. ROSE, In *Pharmacology of Histamine Receptors* (C. R. Ganellin and M. E. Parsons, Ed.), pp. 351–391, Wright-PSG, London, 1982.
27. J. M. ARRANG, M. GARBARG, and C. J. SCHWARTZ, Autoregulation of histamine release in brain by presynaptic H3-receptors, *Neuroscience* **15**, 553–562 (1985).
28. G. NISTICO, D. ROTIROTI, A. DESARRO, F. NACCARI, and J. D. STEPHENSON, Central effects of histamine H1 and H2 receptor agonists and antagonists after intraventricular infusion in fowls, *Res. Commun. Chem. Pathol. Pharmacol.* **27**, 431–450 (1980).
29. B. N. DHAWAN, R. SHUKLA, and R. C. SRIMAL, Analysis of histamine receptors in the central thermoregulatory mechanism of mastomysnatalensis, *Br. J. Pharmacol.* **75**, 145–149 (1982).
30. A. PILC and J. Z. NOWAK, The hyperthermic phase of histamine action in rats, *Pol. J. Pharmacol.* **32**, 469–473 (1980).
31. J. BLIGH, A. SILVER, and C. A. SMITH, The effects on thermoregulatory mechanisms produced by intracerebroventricular injections of histamine in the sheep, *J. Thermal Biol.* **5**, 41–51 (1980).
32. L. S. PELLIGRINO, A. S. PELLIGRINO, and A. J. CUSHMAN, *A Stereotaxic Atlas of the Rat Brain*, Plenum, New York, 1979.
33. D. D. METCALF, M. KALINER, and M. A. DONLON, In *CRC Critical Reviews in Immunology* (M. Z. Atassi, Ed.), pp. 23–74, CRC Press, Boca Raton, FL, 1981.
34. T. F. DOYLE, and T. A. STRIKE, Radiation-released histamine in the rhesus monkey as modified by mast-cell depletion and antihistamine, *Experientia* **33**, 1047–1048 (1977).

35 A. GOTH, In *Medical Pharmacology*, 11th ed. C. V. Mosby, St. Louis, 1984.

36 W. FELDBERG and A. S. MILTON, In *Inflammation* (J. R. Vane and S. H. Ferreria, Eds.), pp. 617–656. Springer-Verlag, New York, 1978.

37 R. UENO, S. NARUMIYA, T. OGOROCHI, T. NAKAYAMA, Y. ISHIKAWA, and O. HAYAISHI, Role of prostaglandin D2 in the hypothermia of rats caused by bacterial lipopolysaccharide. *Proc. Natl. Acad. Sci. USA* 79, 6093–6097 (1982).

38 J. H. SANNER, Antagonism of prostaglandin E2, by 1-acetyl-2-(8-chloro-10,11-dihydrobenyl[1,f][1,4] oxazepine-10 carbonyl hydrazine (SC-19220). *Arch. Int. Pharmacodyn. Ther.* 180, 46–56 (1969).

39 J. H. SANNER, Substances that inhibit the actions of prostaglandins. *Ann. Intern. Med.* 133, 133–146 (1974).

40 H. LABURN, D. MITCHELL, and C. ROSENDORFF, Effects of prostaglandin antagonism on sodium arachidonate fever in rabbits. *J. Physiol. Lond.* 267, 559–570 (1977).

41 S. B. KANDASAMY and B. A. WILLIAMS, Prostacyclin-induced hyperthermia: Implication of a protein mediator. *Neuropharmacology* 21, 1065–1072 (1982).

42 T. F. DOYLE, J. E. TURNS, and T. A. STRIKE, Effect of an antihistamine on early transient incapacitation of monkeys subjected to 4 rads of mixed gamma-neutron radiation. *Aerospace Med.* 42, 400–403 (1971).

43 E. C. LASSER and K. W. STENSTROM, Elevation of circulating blood histamine in patients undergoing deep roentgen therapy. *Am. J. Roentgenol.* 72, 985–988 (1954).

44 L. G. COCKERHAM, T. F. DOYLE, M. A. DONLON, and E. A. HILGERSON, Canine post-radiation histamine levels and subsequent response to compound 48/80. *Aviat. Space Environ. Med.* 55, 1041–1045 (1984).

45 L. G. COCKERHAM, T. F. DOYLE, M. A. DONLON, and C. J. GOSSETT-HAGERMAN, Antihistamines block radiation-induced intestinal blood flow in canines. *Fund. Appl. Toxicol.* 5, 597–604 (1985).

46 V. D. EISEN and C. M. WILSON, The effect of β-irradiation on skin histamine and vascular responses in the rat. *J. Physiol.* 136, 122–130 (1957).

47 H. M. ADAMS and H. K. A. HYE, Concentration of histamine in different parts of cat and its modification by drugs. *Br. J. Pharmacol.* 28, 137–152 (1966).

48 J. F. LIPINSKI, H. SCHAUMBURG, and R. J. BALDESSARINI, Regional distribution of histamine in human brain. *Brain Res.* 52, 403–408 (1973).

49 S. H. SNYDER and K. M. TAYLOR, Histamine in the brain: A neurotransmitter. In *Perspectives in Neuropharmacology* (S. H. Snyder, Ed.), pp. 43–73. Oxford Univ. Press, New York, 1972.

50 J. C. SCHWARTZ, Histaminergic mechanisms in brain. *Annu. Rev. Pharmacol. Toxicol.* 17, 325–338 (1977).

51 M. BAUDRY, M. P. MARTIS, and J. C. SCHWARTZ, The subcellular localization of histamine decarboxylase in various regions of rat brain. *J. Neurochem.* 21, 1301–1309 (1973).

52 M. BAUDRY, M. P. MARTIS, and J. C. SCHWARTZ, H1 and H2 receptors in the histamine-induced accumulation of cyclic AMP in guinea pig slices. *Nature (London)* 253, 362–364 (1975).

53 K. M. TAYLOR and S. H. SNYDER, Brain histamine: Rapid apparent turnover altered by restraint and cold stress. *Science* 172, 1037–1039 (1971).

54 P. LOMAN and M. D. GRUEN, Histamine. In *Body Temperature Regulation, Drug Effects and Therapeutics Implications* (P. Lomax and H. Schonbaum, Eds.), pp. 289–309. Dekker, New York, 1979.

55 L. EDVINSSON, J. CERVOS-NAVARRO, L. LARSSON, C. H. OWMAN, and A. L. RONNBERG, Regional distribution of mast cells containing histamine, dopamine, or 5-hydroxytryptamine in the mammalian brain. *Neurology* 27, 878–883 (1977).

56 P. M. GROSS, Cerebral histamine: Indications for neuronal and vascular regulation. *J. Cereb. Blood Flow Metabol.* 2, 3–23 (1982).

57 H. KAWABE, H. HAYASHI, and O. HAYAISHI, Differential calcium effects on prostaglandin D2 generation and histamine release from isolated rat peritoneal mast cells. *Biochem. Biophys. Res. Commun.* 143, 467–474 (1987).

58 R. W. FULLER, C. S. DIXON, C. T. DOLLERY, and P. J. BARNES, Inhaled prostaglandin D2 potentiates histamine induced bronchoconstriction. *Thorax* 39, 699–700 (1984).

59 R. J. FLOWER, E. A. HARVEY, and W. P. KINGSTON, Inflammatory effects of prostaglandin D2 in rat and human skin. *Br. J. Pharmacol.* 56, 229–233 (1976).

RADIATION RESEARCH 121, 71–75 (1990)

Trehalose Dimycolate Enhances Survival of Fission Neutron-Irradiated Mice and *Klebsiella pneumoniae*-Challenged Irradiated Mice[1,2]

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McCHESNEY, D. G., LEDNEY, G. D., AND MADONNA, G. S. Trehalose Dimycolate Enhances Survival of Fission Neutron-Irradiated Mice and *Klebsiella pneumoniae*-Challenged Irradiated Mice. *Radiat. Res.* 121, 71–75 (1990).

The survival of B6D2F1 female mice exposed to lethal doses of fission neutron radiation is increased when trehalose dimycolate (TDM) preparations are given either 1 h after exposure or 1 day before exposure to radiation. TDM in an emulsion of squalene, Tween 80, and saline was the most effective formulation for increasing the 30-day survival of mice when given 1 day before (90%) or 1 h after (88%) exposure to radiation. An aqueous suspension of a synthetic analog of TDM was less effective at increasing 30-day survival (60%) when given 1 day prior to radiation exposure and not effective when given 1 h after radiation. Mice receiving a sublethal dose (3.5 Gy) of fission neutron radiation and either the TDM emulsion or synthetic TDM 1 h after irradiation were substantially more resistant to challenge with 10, 100, 1000, or 5000 times the $LD_{50/30}$ dose of *Klebsiella pneumoniae* than untreated mice. © 1990 Academic Press, Inc.

INTRODUCTION

The effects of radiation on mammalian hematopoietic, myelopoietic, and gastrointestinal systems are diverse and depend on both the exposure dose and the quality of radiation. Several studies have focused on the effects of X rays (*1–4*) and ^{60}Co γ rays (*5, 6*) on these systems in mice. Other studies have examined the effect various specific and nonspecific immunomodulators have on survival in mice given ^{60}Co γ rays (*7, 8*). However, there are a limited number of reports (*5, 9, 10*) on the effects of fission neutron radiation on these systems and the effect immunomodulators have on these animals' survival.

[1] Supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under Research Work Unit 00129. The views presented in this paper are those of the authors. No endorsement by the Defense Nuclear Agency has been given or should be inferred.

[2] Research was conducted according to the principles enuciated in the "Guide for the Care and use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council.

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Nuclear weapons detonations or nuclear criticality accidents can produce mixed radiation fields of various proportions of photon and neutron radiations. Therefore, it is important to determine the response of animals to mixed-field radiation in order to evaluate realistically the effect cytokines and immunomodulators of nonspecific resistance to infection might have on the animals' survival. Several different formulations of the immunomodulator trehalose dimycolate (TDM) have been shown to be effective in increasing survival of mice exposed to ^{60}Co γ rays (*7*). Therefore it is of interest to evaluate TDM formulations for their ability to increase survival in animals irradiated with mixed-field fission neutrons.

Exposure to fission neutron radiation is more effective in causing severe leukocytopenia in mice within 4 days than exposure to ^{60}Co γ radiation (*11, 12*). Hemopoietic recovery occurs in animals exposed to 7.0 Gy ^{60}Co radiation by Day 14 after exposure (*13, 14*). Mice exposed to 3.5 Gy fission neutrons recover in a similar time period. Exposure to higher levels of radiation produces leukocytopenia and irreversible gastrointestinal damage. Death normally occurs in these mice in less than 14 days due to denudation of the intestinal mucosa, fluid and electrolyte imbalance, and bacteremia (*15*). Infection is a major cause of death in animals exposed to radiation doses sufficient to depress the immune system severely but not to produce irreversible gastrointestinal damage, for example, 5.75 Gy fission neutrons or 10.5 Gy ^{60}Co. The source of the infection can be translocation of normal intestinal flora or an external source. In these circumstances, bacteria of the family Enterobacteriaceae often become opportunistic pathogens. One member of this family, *Klebsiella pneumoniae*, is associated with a high incidence of mortality in immunocompromised patients (*16, 17*).

In this paper, we report on the effects of TDM on the survival of fission neutron-irradiated mice when TDM is used as a protectant (before exposure) and as a therapeutic agent (after exposure) in irradiated mice. We also report the effects of TDM as a therapeutic agent for irradiated mice challenged with *K. pneumoniae*.

MATERIALS AND METHODS

Mice. JAX:B6D2F1 female mice, 12–15 weeks of age (20–25 g), were quarantined on arrival and screened for evidence of disease before being

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released for experimental use. They were maintained in an AAALAC-accredited facility in plastic Micro-Isolator cages containing autoclaved hardwood chip, contact bedding. Mice were provided commercial rodent chow and acidified tap water (pH 2.5 with concentrated HCl) *ad libitum*. Animal rooms were maintained at 70 ± 2°F with 50 ± 10% relative humidity using at least 10 air changes per hour of 100% conditioned fresh air. The mice were on a 12-h light/dark full spectrum lighting cycle with no twilight. All research was conducted in accordance with NIH and our Institutional Animal Care and Use Committee guidelines for the care and use of laboratory animals.

Radiation. The techniques and dosimetry of exposing mice to mixed radiation fields produced by the AFRRI TRIGA reactor were previously described (*18*). All radiation doses reported in this paper are the midline tissue dose as measured using ionization chambers (*19*). In the present study, a neutron to γ kerma ratio of 1:1 (N:G = 1) at midline in the animals was achieved by using a 15.2-cm lead shield in front of the reactor wall. The neutron to γ-ray kerma was chosen as representative of conditions which might prevail during a nuclear weapons detonation or nuclear criticality incident. Mice challenged with *K. pneumoniae* were given a nonlethal total (neutron plus γ-ray) dose of 3.5 Gy midline tissue dose at 0.4 Gy/min. Mice not challenged with *K. pneumoniae* received 5.75 Gy; this is the radiation dose that kills 80% of the mice of this strain receiving no supportive therapy within 30 days ($LD_{80/30}$). Mice were exposed individually in well-ventilated aluminum restraining tubes that rotated at 1.5 rpm.

Dose reduction factor. The dose reduction factor was determined for irradiated mice receiving intraperitoneal (ip) injections of the various TDM formulations or control formulations either 1 day before or 1 h after irradiation with fission neutrons. Groups of 10 mice were exposed to increasing doses of radiation and their 30-day survival was monitored. Probit analysis of the survival data was used to determine the best fit, and thus the $LD_{5/30}$ to $LD_{95/30}$ values, and to determine the dose reduction factor (DRF).

Immunomodulators. Synthetic trehalose dimycolate (S-TDM), a product containing corynomycolic acid and trehalose, and native TDM in a mixture of 2% squalene and 0.2% Tween 80 were produced by Ribi ImmunoChem Research Inc. (Hamilton, MT). S-TDM was prepared as previously described (*7, 20*). The native TDM was suspended in saline to give a native TDM-squalene-Tween 80-saline emulsion (TDM-O). The concentration of both TDM formulations was 200 μg TDM/ml. Controls for these preparations were 0.2% Tween 80-saline (TS) and 2% squalene in 0.2% TS (squalene emulsion). Mice received intraperitoneal injections of 0.5 ml of the appropriate TDM formulation or control formulations.

Bacteria. A clinical isolate of *K. pneumoniae*, serotype 5, was prepared as previously described (*7*). The pellet was washed twice with cold saline and suspended to an optical density at 650 nm, known to yield 1×10^9 viable bacteria/ml. The actual number of viable bacteria was determined by plate counts on Trypticase Soy Agar (BBL, Cockeysville, MD). Dilutions for injection into mice and plate counts were made in sterile saline.

Bacterial $LD_{50/30}$. The dose of *K. pneumoniae* lethal to 50% of mice within 30 days ($LD_{50/30}$) was determined for control and irradiated mice by subcutaneously injecting 10-fold dilutions of *K. pneumoniae* (10^1–10^8 bacteria/0.1 ml) and monitoring survival for 30 days. Mice were injected with bacteria on Days 1, 4, 7, 10, and 14 postirradiation. To assure precise delivery of bacteria and prevent injury to the animal, mice were anesthetized by inhalation of methoxyflurane prior to injection of bacteria. Eight mice were used for each treatment group and a total of six bacteria challenge concentrations were used to determine each $LD_{50/30}$ end point for irradiated mice; four challenge concentrations were used for unirradiated control mice. Groups of irradiated mice challenged on Days 1, 4, and 7 received 10^1, 10^2, 10^3, 10^4, 10^5, or 10^6 CFU/mouse. Irradiated groups challenged on Days 10 and 14 received 10^3, 10^4, 10^5, 10^6, 10^7, or 10^8 CFU/mouse. Groups of unirradiated control mice were injected with 10^5, 10^6, 10^7, or 10^8 CFU/mouse.

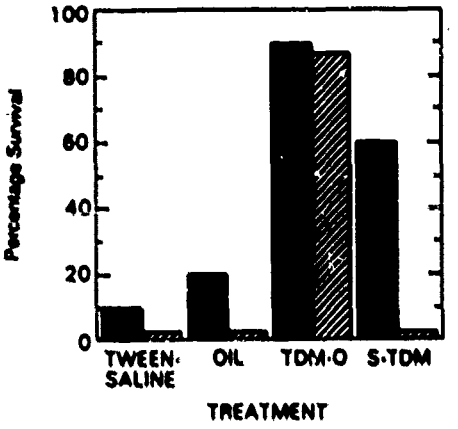

FIG. 1. Percentage 30-day survival of B6D2F1 female mice that received 5.75 Gy, N:G = 1 ($LD_{80/30}$), and 0.5 ml of Tween-saline, squalene emulsion, TDM-O, or S-TDM. The above formulations were injected ip into groups of 10 mice either 1 day before (filled) or 1 h after (hatched) radiation exposure.

Hematology. Mice received either 3.5 or 5.75 Gy of mixed-field radiation. Experimental groups received either 100 μg/0.5 ml S-TDM or 0.5 ml of 0.2% Tween-saline 1 h postirradiation. Mice were anesthetized by inhalation of methoxyflurane immediately before blood was obtained by cardiac puncture. Immediately after blood was drawn, mice were euthanized by CO_2 inhalation. Hematology studies were performed by certified technicians of the Hematology section of our Institute's Veterinary Sciences Department.

RESULTS

In a series of protection experiments, mice were injected ip with the TDM preparations 1 day prior to exposure to a 5.75-Gy MLT dose of fission neutron radiation. TDM-O provided greater protection (90%) than S-TDM (60%), while 10% of mice receiving Tween-saline survived and 20% of those receiving the 2% squalene emulsion survived (Fig. 1). The TDM-O ($P < 0.01$) and S-TDM ($P < 0.05$) were significantly better than TS at increasing 30-day survival of the mice exposed to radiation. The TDM-O was significantly better ($P < 0.01$) than the 2% squalene emulsion.

When the TDM preparations were given therapeutically 1 h after exposure to 5.75 Gy fission neutron radiation, TDM-O provided the most benefit (88%) (Fig. 1). The TDM-O formulation was significantly better than the squalene emulsion alone ($P < 0.001$) when given 1 h after radiation. No mice survived in the groups receiving S-TDM, TS, or 2% squalene emulsion. Incremental increases in the amount of S-TDM given up to 800 μg/mouse did not significantly increase the survival of mice when given 1 h after mixed-field radiation over that observed with 100 μg/mouse (data not shown).

Dose reduction factors were determined for irradiated mice which received S-TDM, TDM-O, Tween-saline (con-

TABLE I
Probit Analysis of Mortality Data for Mixed-Field Irradiated Mice Receiving Formulations Pre- or Postirradiation

Time of treatment	Vehicle	Treatment	$LD_{50/30}$ (Gy)	DRF*
1 Day	Tween-saline	Tween-saline	530	1.00
	Tween-saline	S-TDM	569	1.07
	Squalene emulsion	Squalene emulsion	555	1.00
	Squalene emulsion	TDM-O	596	1.07
	Tween-saline	None	542	0.98
+1 h	Tween-saline	Tween-saline	514	1.00
	Tween-saline	S-TDM	548	1.07
	Squalene emulsion	Squalene emulsion	540	1.00
	Squalene emulsion	TDM-O	581	1.08
	Tween-saline	None	542	0.95

Note. Dose-response survival factors in irradiated mice receiving TDM formulations. B6D2F1 mice received increasing amounts of mixed radiation (N:G = 1). One day before or 1 h after irradiation, groups of 20 mice received 0.5 ml of one of the following formulations (Treatment) by ip injection: Tween-saline, 2% squalene emulsion, 100 μg TDM-O, or 100 μg S-TDM. Mice used in this experiment received only one ip injection; the formulation in the Vehicle column was not injected unless it also appears in the Treatment column. Survival was followed for 30 days. Probit analysis of the results showed the slopes of the probit lines in the comparisons to be parallel; all reported DRFs are based on the $LD_{50/30}$ radiation dose for ease of comparison.

*DRF = $LD_{50/30}$ vehicle.

trol), or the squalene emulsion (control) (Table I). Probit analysis of the results showed the slopes of the probit lines in the comparisons to be parallel; all DRFs are reported at the $LD_{50/30}$ radiation dose for ease of comparison. For both time courses, the DRFs for TDM-O and S-TDM were similar when compared to the appropriate controls. Tween-saline lowered the DRF below that of irradiated animals not receiving injections.

The bacterial $LD_{50/30}$ for mice exposed to 3.5 Gy fission neutron radiation and challenged with *K. pneumoniae* fell by 5 orders of magnitude by Day 4 and remained depressed (2.5 orders of magnitude) on Day 7 (Fig. 2). On Day 10 postirradiation the increased $LD_{50/30}$ indicated that the mice were recovering. By Day 14 the $LD_{50/30}$ returned to nearly that of controls (overlapping 95% confidence intervals). These data indicate that the animals are at greatest risk between Days 1 and 7 postirradiation because it is during this time that an exogenous bacterial challenge has the greatest effect on mortality.

The TDM formulations given 1 h after exposure to 3.5 Gy fission neutron radiation had an effect on the 30-day survival of mice challenged with 10, 100, 1000, or 5000 times the $LD_{50/30}$ dose of *K. pneumoniae* on Day 4 after exposure to radiation (Fig. 3). At the relatively low challenge dose of 10 times the $LD_{50/30}$, all of the TDM formulations increased 30-day survival, as did the squalene emulsion. When the challenge dose of *K. pneumoniae* was increased to 100 times the $LD_{50/30}$, only the TDM-O, S-TDM, and squalene emulsion increased the number of mice surviving over that of the control. The therapeutic effect provided by the squalene emulsion was not significantly ($P > 0.25$) different than control. At 1000 and 5000 times the $LD_{50/30}$ dose of *K. pneumoniae*, only TDM-O and S-TDM

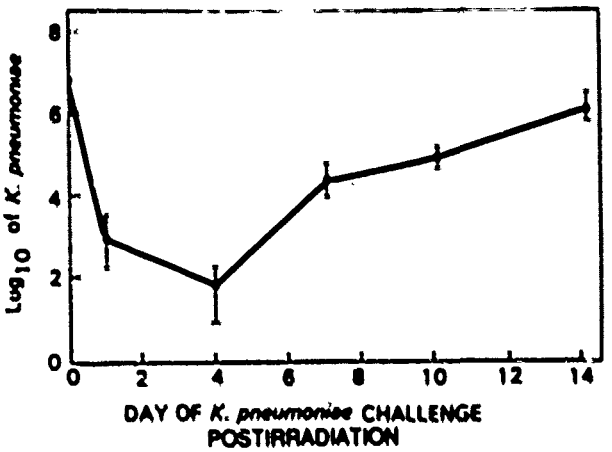

FIG. 2. Bacterial $LD_{50/30}$ of mice challenged with *K. pneumoniae* following radiation exposure. B6D2F1 mice were given 3.5 Gy (N:G = 1) radiation. *K. pneumoniae* was injected into groups of eight mice on Days 1, 4, and 7 (10^1, 10^2, 10^3, 10^4, 10^5, 10^6 CFU/mouse); and on Days 10 and 14 (10^3, 10^4, 10^5, 10^6, 10^7, 10^8 CFU/mouse). Groups of eight unirradiated control mice were injected with 10^1, 10^4, 10^7, or 10^8 CFU/mouse. The $LD_{50/30}$ was determined by probit analysis and plotted for each time of injection after radiation. Vertical bars represent the upper and lower 95% confidence limits for each $LD_{50/30}$.

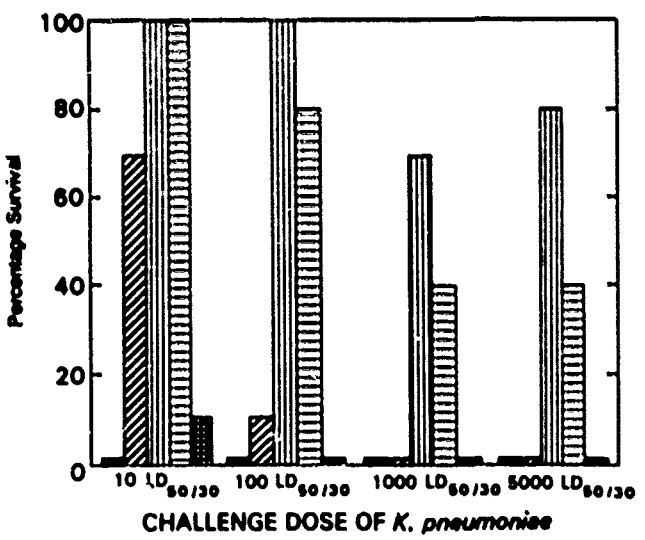

FIG. 3. Thirty-day survival of B6D2F1 female mice given 3.5 Gy (N:G = 1) radiation and treated with TDM prior to challenge with *K. pneumoniae*. One hour after irradiation, 0.5 ml of one of the following was injected ip into groups of 20 mice: (▩) Tween-saline; (▩) squalene emulsion; (▩) 100 mg TDM-O; (▩) 100 mg S-TDM; (▩) controls. Mice were challenged on Day 4 after irradiation with either 10, 100, 1000, or 5000 times the $LD_{50/30}$ of *K. pneumoniae* serotype 5.

increased survival. TDM-O increased survival to twice that of the S-TDM-treated group. The $LD_{80,48}$ (i.e., 80% survival) for unirradiated mice is 2.08×10^6 with a lower confidence limit of 7.95×10^5, while for the irradiated mice treated with TDM-O the corresponding $LD_{80,48}$ is 4.38×10^5. Thus TDM-O is capable of increasing the irradiated mouse's resistance to bacterial challenge to nearly that of a normal mouse.

White blood cell (WBC), red blood cell (RBC), and platelet counts were obtained from mice given 3.5 or 5.75 Gy of mixed-field radiation and treated with either 100 μg S-TDM/0.5 ml or an equal volume of the vehicle (0.2% Tween–saline) 1 h postirradiation. White blood cell counts were similar for mice receiving either 3.5 or 5.75 Gy until Day 14 after radiation exposure. At this time the values were significantly lower ($P < 0.05$) for mice exposed to 5.75 Gy (data not shown). This is true whether or not mice received S-TDM 1 h after exposure to radiation.

The effect of the two doses of radiation on the RBC and platelet counts appeared to be similar until Day 14 for mice treated with S-TDM (data not shown). By Day 14, the RBC and platelet counts in mice exposed to 3.5 Gy were recovering, and by Day 28 they were within 20% of unirradiated control values. On Day 14, the surviving mice of the group given 5.75 Gy and S-TDM 1 h after radiation had RBC and platelet levels significantly lower ($P < 0.05$) than those of mice receiving S-TDM 1 h after 3.5 Gy.

DISCUSSION

For an immunomodulator to be useful as a therapeutic or radioprotective agent in the context of mixed-field radiation, it must be effective over a range of neutron to photon ratios and have a low potential for toxicity. Madonna *et al.* (7) showed that TDM and a synthetic analog, S-TDM, are capable of increasing survival in mice exposed to an $LD_{80,48}$ dose of ^{60}Co radiation. Additionally, they showed that TDM-O and S-TDM increase the survival of mice exposed to a sublethal dose of ^{60}Co γ radiation and challenged with *K. pneumoniae* serotype 5. In our experiments, we have used the more severe radiation challenge generated by a mixed radiation field of equal doses of fission neutrons and photons. Our results demonstrate that both TDM-O and S-TDM formulations are effective in increasing survival when given 1 day before exposure to an $LD_{80,48}$ dose of mixed-field radiation. However, only TDM-O is effective at increasing survival at this radiation level when given 1 h after radiation. The inability of S-TDM to increase survival at the $LD_{80,48}$ radiation level when given 1 h after mixed-field radiation is unclear, especially since S-TDM is effective in increasing survival to an equivalent radiation dose when the radiation is only photons (7). The differences observed are most likely due to the slightly different effect each type of radiation has on cells (21). Specifically, the difference might be related to a difference in the ability of each type of radiation, mixed-field or pure γ radiation, to generate oxygen-derived free radicals and hydrogen peroxide that cause peroxidation of membrane-polyunsaturated fatty acids (22). Additionally, squalene, a long-chain, polyunsaturated hydrocarbon, may act as a quencher to destroy free radicals and hydrogen peroxide before these agents can affect membrane-polyunsaturated fatty acids. This concept is consistent with the increased survival observed (Figs. 1 and 3) when squalene alone is given and might also explain the increased effectiveness of the TDM-O formulation.

Synthetic TDM is an effective therapeutic agent for both endogenous and exogenous infections in irradiated mice. Mixed-field radiation doses of either 3.5 or 5.75 Gy both reduce the number of WBCs, RBCs, and platelets on Days 1 through 7 to similar levels. However, with the sublethal dose, recovery of these cellular fractions begins by Day 14, while with the 5.75-Gy dose they remained depressed. If the mice receiving the sublethal dose of radiation are challenged with *K. pneumoniae* on Day 14 after radiation the number of organisms required for an $LD_{80,30}$ is equivalent to unirradiated controls, 1.4×10^7. However, if these mice are challenged on Day 4, the $LD_{80,30}$ is reduced to 81 organisms. If, however, mice receive S-TDM 1 h after irradiation, 40% of those challenged are able to survive 5000 times the $LD_{80,48}$ dose of the organisms. An identical experiment using TDM-O had 80% survival at this level of bacterial challenge. From our results, it is clear that S-TDM is an effective therapeutic agent at moderate mixed-field radiation levels and an effective therapeutic agent at both moderate and high levels of ^{60}Co (7).

Preliminary data suggest that S-TDM increases the amount of tumor necrosis factor (TNF) present in the 24-h supernatants of macrophages isolated from B6D2F1 mice given S-TDM either 1 day before or 1 h after exposure to an $LD_{80,48}$ dose of mixed-field radiation, compared to radiation and S-TDM controls. Measurement of interleukin 1 (IL-1) in the same supernatants showed a decrease in the amount of IL-1 present when S-TDM is given in conjunction with radiation, compared to radiation alone. If these results are confirmed, they suggest that the ratio of TNF to IL-1 may be an important aspect in determining whether mice survive. These results would also be consistent with the work of Neta using γ-irradiated C3H/HEN mice (8) and the work of Cross with unirradiated and bacterial-challenged C3H/HEN mice (23). Furthermore, they would be consistent with the recent finding of Slordal *et al.* (24), who showed that murine rTNF-α given before sublethal irradiation reduced the decline of neutrophils and total blood counts after irradiation and also accelerated the subsequent normalization of peripheral blood cell counts.

In conclusion, both S-TDM and TDM-O offer substantial protection to endogenous infection when given 1 day before high levels of mixed-field radiation but only TDM-

O is effective at this level when given after irradiation. Additionally, both S-TDM and TDM-O are effective in increasing survival in irradiated and bacteria-challenged mice when given 1 h after mixed-field radiation. The use of TDM-O in future experiments because of its greater effectiveness when compared to S-TDM must be weighed against the reported toxicity of native TDM preparations delivered in oil emulsions and the absence of toxicity of synthetic analogs of TDM at equivalent concentrations (25). We are continuing to explore the interaction of different types of radiation and S-TDM stimulation of TNF and IL-1.

RECEIVED: April 21, 1989; ACCEPTED: August 28, 1989

REFERENCES

1. H. QUASTLER, The nature of intestinal radiation death. *Radiat. Res.* 4, 303–320 (1956).
2. F. OTTO and U. PITTER, Mortality response of mice after whole-body exposure to 1.7–5 MeV neutrons and X-rays. *Radiat. Res.* 50, 125–135 (1972).
3. V. P. BOND, Comparison of mortality response of different species to x-rays and fast neutrons. In *Biological Effects of Neutron and Proton Irradiation*, Vol. 2, pp. 365–377. IAEA, Vienna, 1965.
4. A. GIGLI and R. GALLILY, Effect of x-irradiation on various functions of murine macrophages. *Clin. Exp. Immunol.* 16, 643–655 (1974).
5. H. H. VOGEL and D. L. JORDAN, Comparison of damage to the small intestine by fission neutrons or gamma rays from 60-Co. In *Gastrointestinal Radiation Injury* (M. F. Sullivan, Ed.), pp. 327–329. Excerpta Medica, Amsterdam, 1966.
6. V. P. BOND, M. FLIEDNER, and J. O. ARCHAMBEAU, Effects of radiation on the hemopoietic system: The bone marrow syndrome. In *Mammalian Radiation Lethality* (V. Bond, T. Fliedner, and J. Archambeau, Eds.), pp. 159–230. Academic Press, New York, 1965.
7. G. S. MADONNA, G. D. LEDNEY, T. B. ELLIOTT, I. BROOK, J. T. ULRICH, K. B. MEYERS, M. L. PATCHEN, and R. I. WALKER, Trehalose dimycolate enhances resistance to infection in neutropenic animals. *Infect. Immun.* 57, 2495–2501 (1989).
8. R. NETA, J. J. OPPENHEIM, and S. D. DOUCHES, Interdependence of the radioprotective effects of human recombinant interleukin 1 alpha, tumor necrosis factor alpha, granulocyte colony-stimulating factor, and murine recombinant granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 140, 108–111 (1988).
9. J. P. GERACI, K. J. JACKSON, G. M. CHRISTENSEN, P. D. THOWER, and M. S. FOX, Cyclotron fast neutrons RBE for various normal tissues. *Radiobiology* 115, 459–463 (1975).
10. C. P. SIGDESTAD, D. J. GRDINA, A. M. CONNOR, and W. R. HANSON, A comparison of radioprotection from three neutron sources and ^{60}Co by WR-2721 and WR-151327. *Radiat. Res.* 106, 224–233 (1986).
11. S. GLASSTONE and P. J. DOLAN, *The Effects of Nuclear Weapons*, 3rd ed., U.S. Government Printing Office, Washington, DC, 1977.
12. K. F. BAVERSTOCK and P. J. N. D. ASH, A review of radiation accidents involving whole body exposure and the relevance to the LD50/60 in man. *Br. J. Radiol.* 56, 837–849 (1983).
13. M. L. PATCHEN, T. J. MACVITTIE, and L. M. WATHEN, Effects of pre and post-irradiation glucan treatment on pluripotent stem cells, granulocyte, macrophage and erythroid progenitor cells and hemopoietic stromal cells. *Experientia* 40, 1240–1244 (1984).
14. R. I. WALKER, G. D. LEDNEY, and C. B. GALLEY, Aseptic endotoxemia in radiation injury and graft-versus-host disease. *Radiat. Res.* 62, 242–249 (1975).
15. P. GUNTER-SMITH, Effect of ionizing radiation on gastrointestinal physiology. In *Military Radiobiology* (J. J. Conklin and R. I. Walker, Eds.), pp. 135–151. Academic Press, New York, 1987.
16. D. CAVANAGH, R. A. KNUPPEL, J. H. SHEPARD, and R. ANDERSON, Septic shock and the obstetrician/gynecologist. *South. Med. J.* 75, 809–813 (1982).
17. J. J. ZIMMERMAN and K. A. DIETRICH, Current perspectives on septic shock. *Pediatr. Clin. North Am.* 34, 131–163 (1987).
18. D. A. STEWART, G. D. LEDNEY, W. H. BAKER, E. G. DAXON, and P. A. SHEFFIY, Bone marrow transplantation on mice exposed to a modified fission neutron (N/G 30:1) field. *Radiat. Res.* 92, 268–279 (1982).
19. G. H. ZEMAN, M. DOOLEY, D. M. EAGLESON, L. J. GOODMAN, R. B. SCHWARTZ, C. M. EISENHAUER, and J. C. MCDONALD, Intercomparison of neutron dosimetry techniques at the AFFRI TRIGA reactor. *Radiat. Prot. Dosim.* 23, 317–320 (1988).
20. G. J. VOSIKA and G. R. GRAY, Phase I study of iv mycobacterial cell wall skeleton and cell wall skeleton combined with TDM. *Cancer Treat. Rep.* 67, 785–790 (1983).
21. B. A. CARNES, Genetic injury in hybrid male mice exposed to low doses of ^{60}Co gamma-rays or fission neutrons. III. Frequencies of abnormal sperm and reciprocal translocations measured during and following long-term weekly exposure. *Mutat. Res.* 198, 285–294 (1988).
22. G. BATIST, A. RAYNAUD, A. G. KATKI, E. L. TRAVIS, M. C. SHOEMAKER, R. F. GRIFFNE, and C. E. MYERS, Enzymatic defense against radiation damage in mice. Effect of selenium and Vitamin E depletion. *Biochem. Pharmacol.* 35, 601–606 (1986).
23. A. S. CROSS, J. C. SADOFF, N. KELLY, E. BERNTON, and P. GEMSKI, Pretreatment with recombinant murine tumor necrosis/cachectin and murine interleukin-1 alpha protects mice from lethal bacterial infection. *J. Exp. Med.* 169, 2021–2027 (1989).
24. L. SLORDAL, D. J. WARREN, and M. A. S. MOORE, Effect of recombinant murine tumor necrosis factor on hemopoietic reconstitution in sublethally irradiated mice. *J. Immunol.* 142, 833–835 (1989).
25. E. LEDERER, An update on natural and synthetic trehalose-diesters. In *Immunomodulators and Nonspecific Host Defense Mechanisms against Microbial Infections* (K. N. Masihi and W. Lange, Eds.), pp. 73–83. Pergamon, New York, 1988.

Brain Research, 509 (1990) 280–292
Elsevier

BRES 15208

Neural grafts attenuate behavioral deficits produced by early radiation-induced hypoplasia of fascia dentata granule cells

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(Accepted 8 July 1989)

Key words Ionizing radiation; Rotation; Behavior, Passive avoidance; Transplant; Neural graft; X-ray; Hippocampus; Fascia dentata; Granule cell; Spontaneous alternation

Localized X-irradiation of the mitotic cells in the neonatal rat hippocampus produces a discrete hypoplasia of the fascia dentata granule cell layer. This brain damage inhibits the acquisition of a passive avoidance task, and stimulates spontaneous perseverative turning (without reversals) in a plastic hemisphere apparatus. Here we report how transplantation of fetal brain tissue can attenuate these radiation-induced behavioral deficits. The partially shielded cerebral hemispheres of neonatal rats received fractionated exposures to 13 Gray (Gy) of X-rays during the first 16 days post partum. This procedure depleted 90% of the hippocampal granule cells while sparing other brain areas. Control animals were sham irradiated. Baseline behavioral tests were conducted when subjects reached an average age of 147 ± 4 days. We recorded behavioral parameters known to be sensitive to hippocampal damage: (1) passive avoidance performance, and (2) perseverative spontaneous turning without reversals. Irradiated subjects later (average age = 182 ± 4 days) received intracerebral transplants of either fetal (E20–21) neurons/neuronal precursors from the fascia dentata or cerebral cortex (control grafts). Additional controls (both irradiated and sham-irradiated) experienced sham surgical procedures or received no surgical manipulation. Two post-surgical behavioral retests were accomplished when rats were 265 ± 5 and 351 ± 6 days old. Rats were then sacrificed and brains were treated histologically to assess radiation-induced brain damage, graft survivability and graft locus. Both hippocampal and cerebral cortex grafts generally facilitated performance on the passive avoidance task. This effect was most prominent during the first post-surgical test. Hippocampal transplants (especially those found to reside in the damaged hippocampus) also significantly attenuated perseverative spontaneous rotation at the time of the final post-surgical test series. Cortex grafts found within the damaged hippocampus did not ameliorate perseverative movements, while cortex grafts located outside the hippocampus significantly reduced this behavioral deficiency. These data suggest that selected behavioral deficits may be attenuated by transplanting fetal neural tissue long after early radiation-induced brain damage. The success of these procedures depends on a number of factors including: (1) the behaviors chosen for analysis, (2) the time after transplantation that behavioral tests are conducted, and (3) the source and final location of the donor neural tissue.

INTRODUCTION

A growing literature suggests that brain injuries, once believed to be irreversible, may now be successfully treated through the use of techniques involving neural transplantation of embryonic brain tissue. Such grafts typically exhibit growth, long-term survival and some degree of organotypic differentiation. Further, normalization of neurophysiological, neuroendocrine and behavioral functions have also been reported (for reviews see refs. 3, 13, 46).

By virtue of its precise internal organization and its postulated involvement in learning, memory and other higher cognitive functions[20,31,42,43], the hippocampus has been one of the most extensively investigated neuroanatomical targets in studies involving neural grafting. For example, transplants of septal tissue placed into the site of fimbria-fornix lesions can develop in the host brain and innervate the hippocampus with terminal patterns similar to those in the normal animal[11,12]. These grafts have electrophysiologically functional synapses and they can significantly reduce maze learning deficits usually observed in animals with fimbria-fornix lesions[21,35]. Recently, other investigators have found that transplantation of fetal hippocampal tissue into the site of a hippocampal aspiration lesion can produce partial recovery of spatial maze learning[32] and improve performance on an operant task requiring low response rates[57].

In many brain lesion experiments difficulties in interpretation may arise due to lack of lesion uniformity and the nonspecific collateral brain damage often produced as part of surgical procedures. However, Bayer and Peters[8] have reported that discrete and fairly uniform lesions of the granule cells in the hippocampal dentate gyrus (fascia dentata) may be observed after X-irradiation of the neonatal rat brain. Radiation-induced hypoplasia of the

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granule cells effectively disrupts a major input to the hippocampus by eliminating the target of cells in the perforant path (from entorhinal cortex). Therefore, although the lesion is discrete, it often produces behavioral consequences similar to hippocampectomy[53,54]. Bayer et al.[5] described locomotor hyperactivity, reduced spontaneous alternation in a T maze and retarded acquisition of a passive avoidance task in rats with early radiation-induced damage of the fascia dentata. More recently, we[38,39] replicated and extended this work by revealing that rats with hypoplasia of the fascia dentata granule cells also exhibit perseverative spontaneous movements.

The usefulness of this lesion method in the study of hippocampal transplantation techniques was established by Sunde et al.[52] and later by Zimmer et al.[58] who reported how grafts of fascia dentata granule cells can restore normal neuronal circuitry initially disrupted by early X-ray exposure. In the current experiment we evaluated the ability of granule cell transplants to reduce behavioral dysfunctions associated with radiation-induced hypoplasia of the dentate granule cells. Here we report that hippocampal grafts can reduce perseverative movements and deficits in the acquisition of a passive avoidance task. However, these behavioral benefits depend on a number of factors including: (1) the behaviors chosen for analysis, (2) the time after transplantation that the behavioral tests are conducted, and (3) the neuroanatomical source and final location of the transplanted tissue.

MATERIALS AND METHODS

Subjects

Pregnant rats (Crl: CD(SD)BR) obtained from Charles River Labs, Kingston, NY, and screened for evidence of disease were housed in a facility accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC). Temperature and relative humidity in the animal rooms were held at 19–21 °C and 50 ± 10%, respectively, with 10+ air changes/h. Full-spectrum lighting was cycled at 12 h intervals (lights on at 06.00 h) with no twilight. The 59 male rats used in these experiments came from a total of 23 different litters. On the day of birth (day 1) litters were culled and only 4–8 males/litter were reared together. Based on a random selection process, from 1 to 7 of these rats in each litter were actually used in the experiments reported here. All rats were weaned at the same time (between 23 and 28 days after birth) and then individually housed in micro-isolator, polycarbonate cages on hardwood chip contact-bedding. Rats were given ad-lib Wayne Rodent Blox and acidified water (HCl, pH 2.5 to prevent the spread of *Pseudomonas*). Palatability studies indicate that animals do not prefer tap water to acidified water and that there are no deleterious effects of this water treatment over the lifetime of the subject (for review, see ref. 55).

Irradiation procedure

Subjects from each litter were randomly assigned to either the X-irradiated or the sham-irradiated (control) group. Irradiated rats received collimated X-rays (Phillips Industrial 300 kvp X-ray machine, Phillips, Inc., Mahwah, NJ; configured with 1.5 mm of copper filtration, with a half-value layer of 2.5 mm copper) delivered dorsally, in the coronal plane, through a narrow slot in a loose-fitting whole-body lead shield. X-rays were confined to that area of the head previously determined to contain the hippocampus. Determinations of the location of the hippocampal formation relative to external landmarks (e.g. snout, eyes, ears) were made during preliminary dissections of other neonatal rats. These external landmarks were subsequently used to set the position of the slot in the lead shield during our irradiation procedure. The measurements and anatomical landmarks we used for shield placement corresponded closely to those previously reported by Bayer and Peters[8]. The slot in the shield was the opening between 2 movable lead strips (22.8 cm × 6.8 cm × 2 cm) suspended just above the heads of the rats in the radiation exposure array. The opening extended laterally beyond the full width of each rat head and varied between 5–10 mm in the anterior/posterior plane in order to accommodate the growth of the head/brain over the course of the radiation treatment (see ref. 8 for a complete explanation of this procedure). The irradiated rats were exposed to 2.0 Gray (Gy) on postnatal days 1 and 2 (day of birth = postnatal day 1), and to 1.5 Gy on postnatal days 5, 7, 9, 12, 14, and 16 for a total partial-head-only dose of 13 Gy (1 Gy = 100 rads). Doses were determined by using Exradin 0.05 ml tissue-equivalent ion chambers with calibration traceable to the National Institute of Standards and Technology. X-rays were delivered at a rate of 0.49 Gy/min (total irradiation time = 3.0–4.0 min) at a depth of 2 mm in tissue. The sham-irradiated control rats were restrained for the same time period as the irradiated rats but were not exposed to X-rays.

The entire anterior/posterior extent of the hippocampal formation was irradiated as were brain areas dorsal and ventral to this structure (see ref. 44 for a listing of these other brain areas). Brain structures anterior and posterior to the slot in the lead were shielded. At the time of our postnatal radiation exposures the rat brain contained three remaining populations of dividing (and therefore radiosensitive) cells: neuronal precursors of granule cells in the hippocampus, cerebellum and olfactory bulbs[5,7]. Two of these major neuronal precursor populations (in the cerebellum and olfactory bulbs) were covered by the radio-opaque shielding. Not shielded were the mitotic (radiosensitive) granule cells of the dentate gyrus and the mature neurons in other brain structures residing in the same coronal plane as the hippocampus. This procedure produces selective hypoplasia of granule cells in the dentate gyrus[8,29] while sparing the radioresistant[16,30] mature neurons of other brain structures. The technique has been validated through a variety of neuroanatomical methods[7,29,54]. The rats in the current study were sacrificed at the end of the experiment to allow histological analysis of the brain (see below).

General procedures and experimental groups

Following irradiation, rats were allowed to mature for approximately 4 months (mean age 147 ± 4 (S.E.M.) days) before baseline behavioral testing began. Neural transplantation and sham-surgical procedures were conducted approximately 5 weeks following the baseline tests. Rats were retested approximately 2.8 months (at mean age of 265 ± 5 (S.E.M.) days) and again at 5.6 months (mean age of 351 ± 6 (S.E.M.) days) after the surgical procedures.

Thirty-seven experimental rats were irradiated in order to produce hypoplasia of the fascia dentata granule cells. Rats were later randomly assigned to various surgical conditions. Some of these animals received hippocampal grafts (n = 9) or tissue from a non-homologous CNS area (cerebral cortex) (n = 8). Other irradiated subjects (n = 11) received no neural grafts but underwent a sham-surgical procedure in order to evaluate the effects of our surgical techniques. Additional rats (n = 9) with radiation-induced fascia dentata damage received no surgical treatment. Sham-irradiated control rats also underwent a sham-surgical procedure (n = 15) or experienced no surgery (n = 7).

Behavioral tests

During each test series we recorded performance on 2 primary

behavioral measures: passive avoidance and spontaneous rotation (see below). Rats with fascia dentata granule cell hypoplasia perform poorly on passive avoidance tasks by readily moving into an area in which they have recently been shocked. These subjects also become fixated on some motor response patterns. They exhibit perseverative turning (in a bowl apparatus) and are less likely to reverse their direction of rotation[6,16,39,53,54]. The order of behavioral tests was randomized for each subject within each test series.

We also recorded the spontaneous locomotor activity of subjects during each of the 3 test series (baseline and 2 post-surgical retests). Non-transplanted rats with radiation-induced hippocampal damage exhibited an initial locomotor hyperactivity during baseline testing[15,39]. However, we have found that hyperactivity decreased markedly as the animals matured[39]. Since locomotor hyperactivity spontaneously recovered, this measure proved to be less useful in the evaluation of the transplantation manipulation and has been eliminated from the current discussion.

Spontaneous rotation

Rotation was measured in one of two opaque, sound-insulated, 60 cm diameter plastic hemispheres (bowls). Rotation within the hemisphere was inevitable since this was the primary gross movement permitted by the shape of the apparatus. Circling was measured through a projector-drive cable clipped to a wide rubber band around the rat's thorax. Following the design of Greenstein and Glick[26], the cable turned a slotted illuminated disk uncovering one of 4 phototransistors. The time and direction of each quarter turn in each of 6, 30 min sessions was recorded on a microcomputer.

Rats were tested at approximately the same time during the light portion of the day for 6 consecutive days. After each rat was put into the hemisphere and the cable clipped to its band harness, the hemisphere's sound-attenuating lid was lowered, leaving a 0.5 mm gap. Spontaneous turning was recorded for 0.5 h. Bowls were thoroughly washed between runs. In order to control for an apparatus bias, daily runs were alternated between two test hemispheres.

Passive avoidance

Passive avoidance was measured within a shuttle box comprised of two adjacent compartments (each 22 × 22 × 22 cm) separated by a black plastic guillotine door. Each compartment was made of black plastic except for the top and the front walls which were clear. The goal compartment was identical to the start box except for a small nonoperative light on the wall at the far end and a plastic food tray on this wall 2 cm off the floor. The floor was made of metal rods (0.5 cm diameter, 1.7 cm apart). Leads from a Coulbourn Instrument's (Lehigh Valley, PA) constant-current shocker (Model E13-04) were connected to the floor bars in the goal compartment but not to the bars in the start compartment.

Three to 7 days before training, rats were given daily rations of 5 g of Wayne Rodent Blox and 1-5 g of highly palatable breakfast cereal (Froot Loops, Kellogg Company, Battle Creek, MI). For 2-4 days before training, each rat was allowed to fully explore the shuttle box (with cereal in the food-tray) for 15-45 min. Body weight on the first day of training averaged 87% of weight for the week prior to food reduction. During training (15 trials each day) the rat was placed in the start compartment with the divider door closed. After 5 s the door was raised and a timer started. The trial ended either when the rat grasped a Froot Loop or in 2 min. Between training trials, the rat was returned to its home cage for 30 s. Avoidance testing was started when a rat both averaged less than 10 s per trial to grasp the food during a training session and, on the first 5 trials of the following day, had a median latency to grasp the food of 10 s or less.

Avoidance training/testing consisted of initiating a regular training trial as usual. However, when the rat's 4 feet were in the goal box, the experimenter administered a 0.25 mA scrambled footshock until the rat retreated to the safe side. The rat was then returned to its home cage for 60 s. This procedure was repeated on subsequent trials except that the floor on the food side of the chamber was continuously electrified. The critical latency measured was the time for each rat to cross onto the shock grid. A trial was terminated if the rat either crossed onto the food/shock side of the chamber (registering a decrease in resistance across the rods) or stayed on the safe side for 120 s. The session (and test) was ended when the rat remained in the safe area for 120 s on three consecutive trials or after 20 test trials. After each session the apparatus was thoroughly cleaned and paper under the rod floor was replaced.

Surgery, fetal graft preparation and injection

Fifteen of the sham-irradiated rats and 28 of the irradiated animals underwent a surgical procedure approximately 5 weeks after the baseline behavioral test series (mean age at time of surgery = 182 ± 4 (S.E.M.) days). The animals were injected with atropine sulfate (0.4 mg/kg, i.p.) and then anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Using aseptic techniques, the scalp was opened, a hole was drilled on both sides of the cranium adjacent to the midline, and the dura mater was broken with a probe. In each brain hemisphere a 20 gauge stainless-steel cannula was stereotaxically directed toward the dentate gyrus of the hippocampal formation using the following coordinates: 3.5 mm posterior to bregma; 1.6 mm lateral to the midline; 4.0 mm below the skull[44]. Donor tissues (see below) were cut into approximately 0.027 mm^3 fractions before 3 or 4 of the pieces were suspended in tissue culture medium (Dulbecco's modified Eagle medium, Whittaker M.A. Bioproducts, Walkersville, MD) and injected into each side of the host brain. The total injection volume was 3.0–4.0 μl. Following this procedure the scalp was sutured and subjects were returned to their home cages. Some of our subjects underwent a sham-surgical procedure and were treated as described above except that there was no fetal tissue injected into the brain along with the tissue culture medium. Full recovery was allowed since the next behavioral test series did not commence until almost 100 days after this surgery.

Donor CNS tissue used for transplantation was dissected from E20 to E21 rat fetuses. Each embryo was removed, as needed, while the dam was maintained under deep anesthesia (sodium pentobarbital, 55 mg/kg, i.p.). Following exposure of the brain and spinal cord of the fetuses, these tissues were removed and transferred to sterile tissue culture medium. The meninges and dorsal root ganglia were removed and specific regions of the CNS were dissected using methods similar to those previously described by Stenevi et al.[51]. Donor tissue was taken from either the dentate gyrus of the hippocampus or from the cerebral cortex. The hippocampal tissue used for transplantation consisted predominantly of the granule cells (and precursors) from fascia dentata. However, interneurons and cells from CA3 were also present in many of the grafts.

Histology

After behavioral testing was completed, our rats were anesthetized and perfused with heparinized saline followed by 10% buffered formalin. Brains were embedded in paraffin, serially-sectioned (6 μm) (in either the coronal or sagittal plane) and then stained with Cresyl violet and Luxol fast blue[34]. The brains of all subjects receiving neural transplants were reviewed by one of us (G.A.M.) who was blind to the behavioral results. During this review we confirmed the presence/absence of the grafts, evaluated graft morphology and determined the neuroanatomical location of the neural transplants.

All brains also received a preliminary review to confirm radiation-induced damage to the dentate gyrus. In addition, several (irradiated, $n = 20$; sham-irradiated, $n = 20$) non-transplanted brains were randomly selected, sliced in sagittal section, and analyzed in more detail. A single section (approx. 1.9 mm lateral to the midline)[44] was used for this analysis to (1) estimate the degree of fascia dentata injury and (2) survey the other brain areas (olfactory bulb and cerebellum) that, although shielded from irradiation, are known to contain granule cells mitotic at the time of radiation treatment. We counted the total number of granule cells that could be visualized in the single section of the dentate gyrus used in this analysis. Cell counts were accomplished under 250× total magnification by a

single observer. Nuclear cell counts were used in order to avoid the error caused by double or triple nucleoli. The size, cytoplasmic staining and nuclear structure of granule cells usually makes them distinguishable from glial cells[4,10]. However, the possibility cannot be ruled out that some of the astroglial cells may have also been counted. The impact of this possible error is reduced by the fact that the number of glial cells in the fascia dentata is extremely low[33]. In addition, after neonatal irradiations similar to those described here,

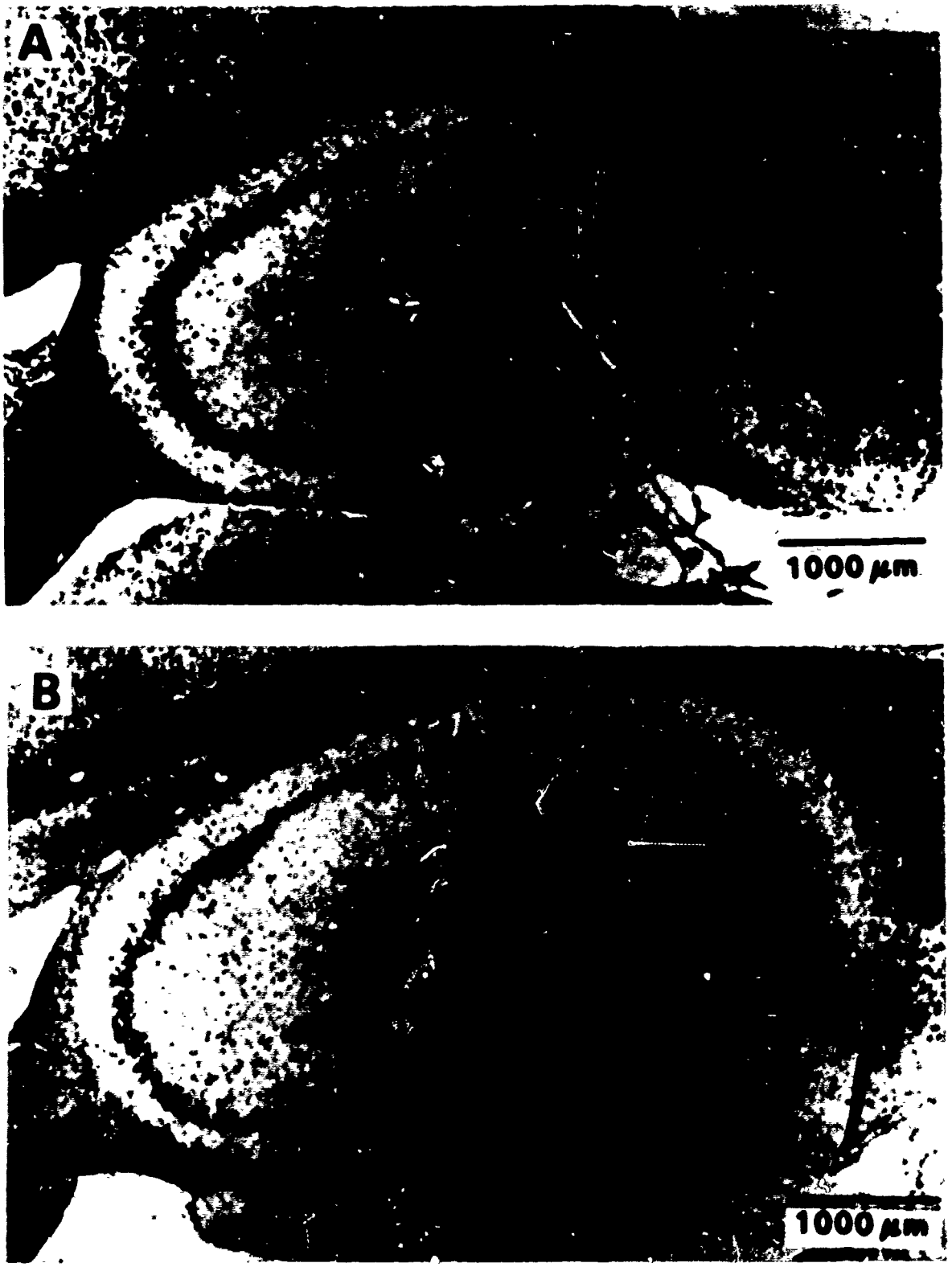

Fig. 1. Representative sagittal sections (approximately 1.9 mm lateral to the midline) from a rat with X-ray-induced hypoplasia of fascia dentata granule cells (A) or a sham-irradiated control rat with an intact dentate gyrus (B).

Bayer and Altman[6] reported that the granule cell population remains significantly reduced into adulthood while the glia show an initial reduction in number followed by a complete regeneration to normal levels within 60–90 days[7]. Thus our cell counts in the fascia dentata of the 1-year-old adult rat would presumably not reflect a radiation-induced alteration in glial population. Using an imaging system (Bioquant System IV, R&M Biometrics, Inc., Nashville, TN) we also derived the area of the dentate gyrus, computed the cellular density of the structure and the thickness of the granule cell layer. In order to confirm that the shielding of other brain areas was sufficient, we also counted granule cells in a 0.004 mm^2 area in the cerebellum and olfactory bulb. Further, we evaluated the sparing of another more mature, and therefore less radiosensitive, hippocampal structure by counting the thickness of the CA1 pyramidal cell layer that was dorsal to the dentate and directly in the path of the X-radiation.

Brains with neural grafts were further analyzed by estimating the volume of the transplants. Using the Bioquant Imaging System, we made measurements of graft areas within every eighth brain section throughout the full extent of the transplants. Estimates of graft volumes were calculated by multiplying the area of the transplant (visualized in each section) by the thickness of tissue between the sections sampled (7 sections × 6 μm section + 42 μm) and then summing these scores for each graft. When more than one transplant was visualized in a particular brain, the individual graft volumes were added to get a total transplant volume for that subject.

Data analysis

In both of our behavioral tests irradiated and sham-irradiated subjects that experienced the sham-surgical procedure were not significantly different (*t*-tests) than rats with no surgical history. Therefore, sham-surgery and no-surgery treatment groups were combined for all statistical analyses reported here.

We assessed performance on the passive avoidance task by analyzing the time spent in the safe compartment before moving onto the shock grid. Of primary interest were latencies on the second trial during the test day since this was the first avoidance trial. Long latencies to move on the trial immediately following the initial footshock reflected superior passive avoidance performance.

Spontaneous perseverative rotation was quantified by recording the bout length each time before a reversal in turning direction was made. The mean turning bout length for each of the 6 daily test sessions was computed by dividing the total quarter turns (in either the dominant or non-dominant direction) by the number of bouts of quarter turns (without reversal of direction) in that session. Statistical comparisons were computed using each rat's average bouth length score. This was the mean of the daily bout lengths for the 6 sessions in the test series.

Where appropriate, we accomplished group comparisons within a particular test series by using a one-way analysis of variance (ANOVA) or a *t*-test. When assumptions of homogeneity of variance could not be met by using the raw data we transformed the scores to logarithms. If the ANOVA revealed statistically significant differences ($\alpha = 0.05$) we usually conducted a priori paired comparisons using *t*-tests[44].

RESULTS

Histological confirmation of fascia dentata granule cell hypoplasia

Exposure of a portion of the neonatal cerebral hemispheres to early, fractionated doses of ionizing radiation produced a selective reduction in granule cells of the hippocampal dentate gyrus while sparing other brain areas (see Fig. 1 and Table I). Specifically, exposure of the neonatal rat hippocampus to ionizing radiation produced a statistically-significant 90% ($t_{36} = -22.4$, $P < 0.001$) depletion in the number of dentate granule cells. Similarly, both the areas and the granule cell densities of the irradiated dentate gyri were significantly reduced compared to those of the control rats ($t_{36} = -13.8$, $P < 0.001$ and $t_{36} = -6.6$, $P < 0.001$, respectively). The specificity of this damage is illustrated by the sparing of the pyramidal CA1 neurons that were directly in the path of the X-rays. Irradiation produced no significant change in the thickness of the CA1 pyramidal cell layer, yet the thickness of the dentate granule cell layer was significantly reduced ($t_{36} = -20.0$, $P < 0.001$).

The granule cell populations (i.e. number of cells/unit area) of the olfactory bulb and the cerebellum were not significantly altered by the irradiation treatment (86% and 110% of control, respectively). Correlations were not statistically significant between granule cell densities in the dentate gyrus and those in the cerebellum or olfactory bulb. However, the granule cell densities of the cerebellum and olfactory bulb (both shielded from radiation exposure) were significantly correlated ($r_{18} = 0.58$, $P < 0.01$). These data suggest that the shielding of the olfactory bulb and cerebellum during the irradiation treatment was effective.

Graft survival, location, volume and cytology

Three or 4 pieces of fetal tissue were injected into each hemisphere of our experimental subjects. Since individual grafts appeared to fuse together in some cases, it was often difficult to count the number of surviving pieces of transplanted tissue. Transplanted rats with at least one viable graft were included in the data pool for this paper. Fascia dentata grafts survived in 9 out of 10 rats and

TABLE I

Histological data derived from analysis of sagittal sections of rat brain

Numbers are means and (in parentheses) S.E.M.s.

Anatomical parameter	*Irradiated (n = 20)*	*Sham irradiated (n = 20)*	*% of control*
Number of dentate granule cells	177.7 (9.3)*	1771.5 (90.7)	10%
Dentate area (mm^2)	0.5 (0.02)*	2.2 (0.15)	24%
Density of dentate granule cells ($/mm^2$)	349.1 (28.0)*	824.2 (69.9)	42%
Thickness** of dentate granule cell layer	3.4 (0.2)*	9.3 (0.4)	36%
Thickness** of CA1 pyramidal cell layer	2.6 (0.1)	2.7 (0.1)	96%
Density of olfactory bulb granule cells ($/mm^2$)	10300.0 (695.5)	11983.8 (790.7)	86%
Density of cerebellum granule cells ($/mm^2$)	16850.0 (545.4)	15325.0 (739.8)	110%

* Significantly different (*t*-tests) from sham-irradiated, $P < 0.001$.
** Number of cells.

grafts of cerebral cortex tissue survived in 8 of 10 subjects. The overall survival rate was 85%.

Following histological review of the transplanted brains we divided subjects into 4 categories: (1) animals with hippocampal grafts located either entirely or partially within the hippocampal formation ($n = 6$) (see ref. 44 for the anatomical limits of this structure), (2) animals with hippocampal grafts entirely out of the hippocampal

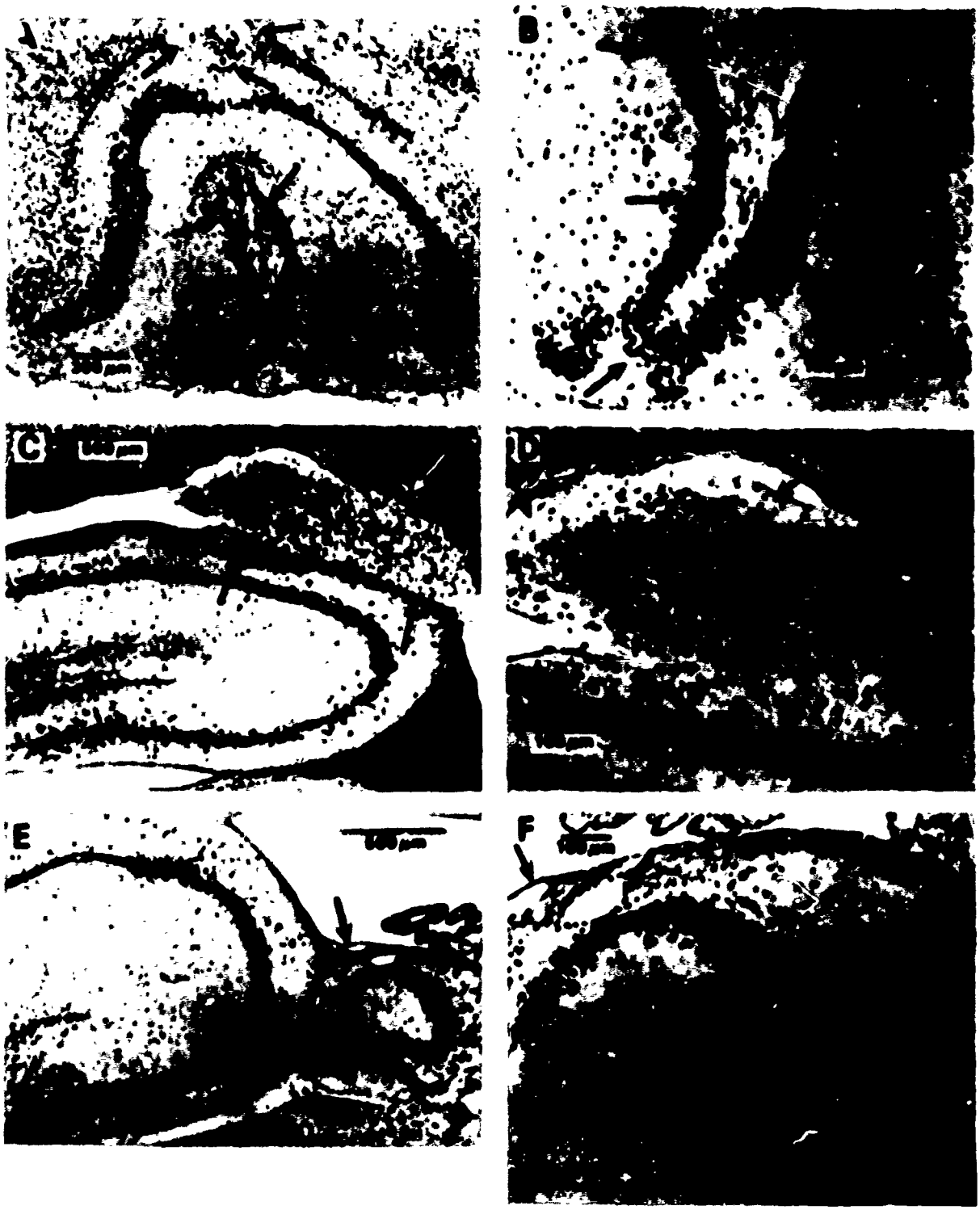

Fig. 2. Examples of fetal brain tissue transplants residing in an adult host brain. Higher magnifications (B,D,F) of the graft in the adjacent section are to the right. Neural grafts from the hippocampus (including fascia dentata granule cells) were placed in the damaged hippocampal formation (A,B). The granule cells in these neuronal grafts often exhibited a crescent-shaped organization of the dentate gyrus (A,B,E,F). On the other hand, the cells in cerebral cortex grafts showed little lamination (C,D). Sometimes both hippocampal tissue transplants (E,F) and cortical grafts (C,D) were found outside of the host hippocampal formation in the lateral ventricle or cerebral cortex. Arrows point to grafted tissue.

formation (n = 3), (3) animals with cerebral cortex grafts located either entirely or partially within the hippocampal formation (n = 3), or (4) animals with cerebral cortex grafts located entirely out of the hippocampal formation (n = 5). These groupings were used to determine how graft location influenced the animal's performance on our behavioral measures.

Our analysis of graft volume revealed that most of our transplants were quite small relative to the graft sizes reported elsewhere[18,19]. Although cerebral cortex grafts tended to be slightly bigger (mean = 0.58 mm^3 ± 0.15 (S.E.M.)) than did fascia dentata transplants (mean = 0.33 mm^3 ± 0.08 (S.E.M.)), this difference in volume was not statistically significant (t-test). Similarly, the volumes of the grafts located inside the hippocampal formation did not differ from those located outside this structure. We found no significant correlations between graft size and post-surgical changes recorded on the spontaneous rotation or passive avoidance measures.

General cytological examination of all of the grafts indicated that they were extensively differentiated and well vascularized. Cerebral cortex transplants showed little internal order or lamination of the neurons and the interiors of the grafts often had white patchy areas free of cells. In the hippocampal grafts, the basic organotypic organization of the main cell and neuropil layers was usually preserved and the characteristic crescent-shaped dentate gyrus was frequently observed (see Fig. 2). Granule cells were most frequently seen in the hippocampal grafts; however, other neurons, including interneurons and pyramidal cells, were located both within and outside the main cell layers. Although efforts were made to restrict placement of fetal tissue to the host hippocampus, there were several instances in which transplants were found in the lateral ventricle or the cerebral cortex. Examination of the extent to which host and graft neuropils were fused indicated that, while close apposition was frequently observed, integration of these natural matrices was only partially achieved. For example, implants in the lateral ventricle were often separated from the host hippocampus by the ependymal lining. However, there were many cases in which denuding of the ependyma had occurred at which points host and graft tissues fused without an intervening cellular partition. Those grafts embedded within the parenchyma of the host hippocampus also exhibited partial integration of the host neuropil, although sometimes regions of the donor tissues periphery were separated from the host brain by scarring or cystic cavities.

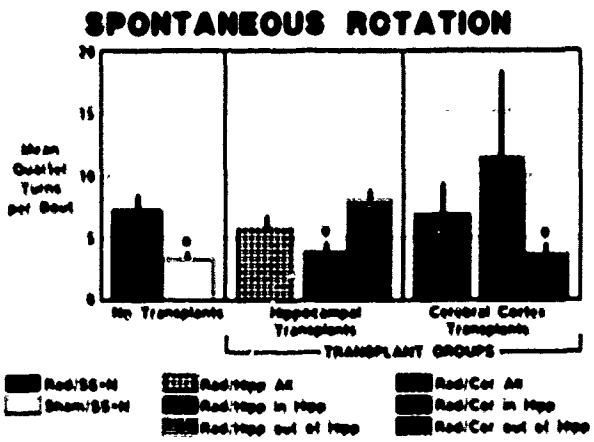

Fig. 3. Length of an average bout of quarter turns during the final postsurgical retest in the plastic hemisphere apparatus. Rats with radiation-induced fascia dentata damage (Rad) and no neural transplants (Rad/SS + N, i.e., combined 'sham surgery' and 'no surgery' subjects; n = 20) exhibited long bouts of circling (without reversals in direction). Control subjects, without brain damage (sham; n = 22), were more likely to break up their movement patterns by reversing direction of movement. Here we show the performance of all subjects with either hippocampal tissue grafts (Rad/Hipp All; n = 9) or cerebral cortex grafts (Rad/Cor All; n = 8). In addition, the adjacent bars represent subcategories of these groups in which the hippocampal (Hipp) or cortical (Cor) transplants were found to be placed either within the hippocampal formation (Hipp in Hipp, n = 6; Cor in Hipp, n = 3) or outside of its anatomical limits (Hipp out of Hipp, n = 3; Cor out of Hipp, n = 5). Both (1) hippocampal tissue grafts found to be located within the hippocampal formation (Rad/Hipp in Hipp), and (2) cerebral cortex tissue transplants located outside of the hippocampus (Rad/Cor out of Hipp), were effective in reducing the spontaneous perseverative turning of rats with fascia dentata damage. * Represents groups significantly different (P < 0.05; see Results section for statistical details) from the rats with fascia damage that had either a sham surgical procedure or experienced no surgery (Rad/SS + N). Variance indicators are the standard errors of the mean (± S.E.M.).

Spontaneous rotation

Baseline data (collected before neural grafting) were analyzed to determine the initial behavioral effects of early fascia dentata granule cell hypoplasia. This analysis involved grouping irradiated or sham-irradiated subjects without regard for their subsequent transplant group assignments. Radiation-induced hippocampal damage caused rats to make long bouts of turns in a plastic hemisphere without reversal of direction (see Fig. 3). Once they began moving, in either direction, irradiated rats perseverated in that turning to an extent significantly greater than the sham-irradiated subjects (t_{73} = 3.86, P < 0.001). Baseline behavioral responses within these irradiated or sham-irradiated groups were fairly homogeneous. This was evidenced by the fact that the perseverative turning responses of rats in the various surgical/graft groups were not significantly different.

During the first post-surgical retest, rats with hippocampal damage, but no neural grafts, continued to exhibit significantly longer turning bouts (mean bout length = 5.32 quarter turns) than did intact controls (mean bout length = 2.7 quarter turns) (t_{51} = 2.92, P < 0.005). Neither hippocampal nor cortical grafts signifi-

cantly altered this response. A paired *t*-test revealed that the mean bout length of non-transplanted X-irradiated rats was significantly higher on the second post-operative test than on the first ($t_{10} = -1.91$, $P < 0.05$). Thus, there existed a time-related potentiation of perseverative movements throughout the course of this experiment (see also ref. 39).

The perseverative turning of rats with hippocampal damage was most apparent during the final behavioral test series (see Fig. 3). Using data from this test we employed an ANOVA to compare the recipients of the hippocampal or cerebral cortex transplants with the irradiated or sham-irradiated controls without grafts ($F_{3,44} = 5.32$, $P = 0.003$). As expected, rats with fascia dentata granule cell hypoplasia had longer bouts of circling than did sham-irradiated controls ($t_{44} = 3.78$, $P < 0.001$). Our analysis of the rotation responses of transplanted subjects was organized into 2 segments. In the first data examination, the behavioral response of rats with hippocampal or cerebral cortex tissue transplants was assessed without recognition of the graft location. While the subjects with hippocampal transplants showed some behavioral recovery, the turning bout lengths of neither animals with hippocampal nor cortical tissue transplants were significantly different from non-transplanted subjects with fascia dentata damage. In the second data treatment, rats in each of our transplant groups were subdivided into those with grafts within the hippocampal formation and those with grafts outside the hippocampal formation (see 'General procedures and experimental groups' section). Rats with hippocampal tissue grafts residing within the hippocampal formation exhibited significantly shorter bouts of turning than did the subjects with ectopic transplants ($t_7 = 4.05$, $P = 0.002$). Further, the mean turning bout length of animals with homotopic grafts was significantly lower than that of irradiated subjects without grafts ($t_{24} = -2.26$, $P < 0.02$)[47] and not different from sham-irradiated controls (see Fig. 3).

The behavioral responses of animals with cerebral cortex transplants within the hippocampal formation were more variable than those of other subjects (see Fig. 3). Although it was not a statistically significant effect, these subjects had longer mean turning bout lengths than did the rats with fascia dentata damage alone. Rats having cerebral cortex tissue grafts located outside the hippocampal formation (usually in the cerebral cortex or lateral ventricles) showed a very different behavioral response. These animals reduced their perseverative turning to levels significantly below those of irradiated rats with no grafts ($t_{23} = -2.34$, $P = 0.003$) and not significantly different from rats with no radiation-induced brain damage. Although the behavioral variability on the spontaneous rotation task was largest in the rats with cortical grafts located within the hippocampus, the range of volumes of these transplants (0.35–0.86 mm^3) was smaller than that determined for grafts found outside the hippocampus (0.17–1.48 mm^3). Thus the variations in cortical graft volume may not adequately account for the behavioral variability observed in the hosts.

We also assessed the role of graft symmetry in functional recovery. Most of our subjects had transplants that survived and grew in both cerebral hemispheres. Only 2 of the rats that received fascia dentata tissue and 3 of the rats receiving cerebral cortex transplants had unilateral grafts. This low *n* makes it difficult to draw any reliable conclusions about the ability of unilateral grafts to promote functional recovery. Therefore we undertook a further analysis that used the volumes of grafts in each hemisphere to determine transplant asymmetries (a ratio) for each subject. Using a regression analysis, graft asymmetries were correlated with our measure of perseveration (mean bout length). This examination of the data failed to reveal a significant relationship between graft asymmetry and perseverative responding. Appar-

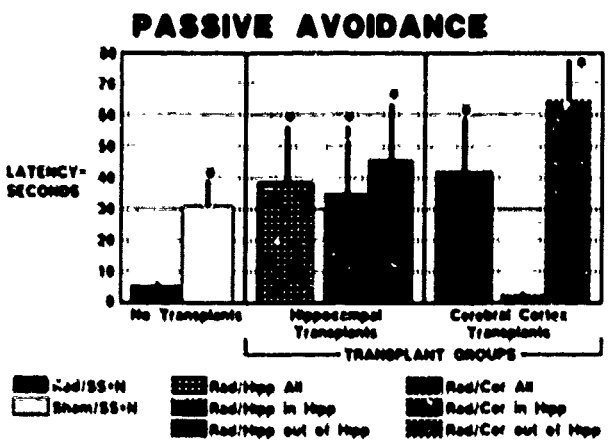

Fig. 4. Passive avoidance performance of both rats with fascia dentata damage (Rad) and sham-irradiated control subjects (Sham) during the first post-surgical retest. These are mean latencies to move out of a safe compartment and into an adjacent area with an electrified grid floor. Rats with hippocampal damage, and no neural tissue grafts (Rad/SS + N, i.e., combined 'sham-surgery' + 'no surgery' subjects; $n = 20$), moved readily into an area where they had just been shocked. Here we show the performance of all subjects with either hippocampal tissue grafts (Rad/Hipp All; $n = 9$) or cerebral cortex grafts (Rad/Cor All; $n = 8$). In addition, the adjacent bars represent subcategories of these groups in which the hippocampal (Hipp) or cortical (Cor) transplants were found to be placed either within the hippocampal formation (Hipp in Hipp, $n = 6$; Cor in Hipp, $n = 3$) or outside of its anatomical limits (Hipp out of Hipp, $n = 3$; Cor out of Hipp, $n = 5$). Independent of their location, hippocampal tissue transplants reduced passive avoidance deficits. On the other hand, cerebral cortex grafts were effective only if they resided outside the hippocampal formation (Rad/Cor out of Hipp). * Represents groups significantly different ($P < 0.05$; Results section for statistical details) from the rats with fascia dentata damage that had either a sham surgical procedure or experienced no surgery (Rad/SS + N). Variance indicators are the standard errors of the mean (S.E.M.).

ently, transplant symmetry plays a minor role in the functional recovery observed in our subjects.

In a final analysis we calculated the turning 'bias' of each animal during each test series. Turning bias is the number of quarter turns in the dominant direction of movement divided by total quarter turns made within the bowl apparatus. Although we always attempted bilateral neural transplants, after histological inspection, we noticed that we could confirm only unilateral grafts in several subjects (see Ns reported above). Since enhanced turning bias can increase the probability of longer bouts of turns without reversals[46] we sought to determine if our unilateral grafts enhanced turning bias. This analysis revealed that grafted subjects did not have greater turning biases than did non-transplanted subjects. Further, rats with unilateral grafts did not have greater turning biases than either irradiated animals with no grafts or irradiated animals with bilateral grafts. Thus, fetal brain tissue transplants can reduce perseverative responses without changing turning bias.

Passive avoidance

During the initial (baseline) assessment of the behavioral deficits following radiation-induced hypoplasia of the fascia dentata granule cells we discovered that the rats with the brain damage tended to move rapidly (mean time 6.9 ± 32 s (S.E.M.)) into an area in which they had just been shocked. Sham-irradiated control rats exhibited significantly longer latencies (mean time 18.1 ± 4.7 s (S.E.M.); $t_{65} = 1.96$, $P = 0.03$)[47]. This difference persisted for non-transplanted subjects during the first post-surgical behavioral retest (see Fig. 4) ($t_{35} = 2.18$, $P < 0.025$). These data may represent a memory deficit. Since spontaneous locomotion of irradiated animals was normal during this test series[39], the finding of shorter latencies to cross onto the shock grid cannot be explained as reflecting a generalized hyperactivity.

Neural transplants produced improved performance on the passive avoidance task. This benefit was most easily discerned during the first behavioral retest following the transplants (approx. 83 days post surgery). Group differences first revealed by a one-way analysis of variance ($F_{3,55} = 2.92$, $P = 0.042$) were further elucidated by paired comparisons using *t*-tests (see Fig. 4). Irradiated subjects with either hippocampal or cortical grafts remained longer on the safe side of the passive avoidance chamber than did the rats with similar brain damage that underwent sham surgery or no surgery ($t_{55} = 2.2$, $P < 0.05$, for hippocampal grafts; $t_{55} = 2.3$, $P < 0.05$, for cortical grafts). During this first post-graft behavioral retest, the transplanted rats with hippocampal damage stayed on the safe side of the passive avoidance apparatus for time periods that were not significantly different from sham-irradiated controls that had no initial brain damage. Behavioral effects of grafts were more difficult to observe on the last passive avoidance test (approx. 169 days after surgery) since there was a tendency for spontaneous improvement in the performance of control rats (i.e. those receiving no surgical treatment or sham surgery) by this time.

We undertook a further analysis of the transplanted subjects' data to determine if the final neuroanatomical location and/or source of the graft tissue had an influence on the behavioral benefits observed on the passive avoidance task. A one-way analysis of variance ($F_{5,53} = 3.02$, $P = 0.018$) compared the latencies to cross onto the shock grid exhibited by irradiated rats having grafts located either within the hippocampal formation or outside of this structure. Transplants found outside the targeted hippocampus were usually in the lateral ventricle or cerebral cortex just adjacent to, and frequently touching, the hippocampus (see Fig. 2). Subsequent *t*-tests revealed that hippocampal granule cell grafts produced similar benefits in passive avoidance performance independent of their location in the brain. Fascia dentata grafts located either within or just outside the hippocampal formation produced similar improvements in passive avoidance as compared to the performance of rats receiving sham surgery or no surgery ($t_{53} = 1.79$, $P < 0.05$). On the other hand, only cortical grafts that were misplaced and located in the host cerebral cortex, had a beneficial effect on passive avoidance responses when compared to non-brain-damaged controls ($t_{53} = 3.28$, $P < 0.01$). Cortical transplants located within the hippocampal formation produced no improvements on this task. Thus fascia dentata granule cells produced an improvement in passive avoidance that was location-independent. Cortical tissue found in a non-homologous location (i.e. hippocampus) did not have this beneficial effect.

DISCUSSION

These experiments extend the studies of Sunde, et al.[52], and Zimmer et al.[58-60] who reported reorganization of the brain after early radiation-induced hypoplasia of hippocampal granule cells and the normalization of neural connections following placement of hippocampal grafts in the dentate gyrus. The results of our study demonstrate several behavioral benefits produced by transplantation of embryonic brain tissue into the brains of rats with similar radiation-induced damage to the fascia dentata granule cells. Here we report that the functional benefits of neural grafts seem to be dependent on a precise combination of conditions rather than being a general phenomenon. The neuroanatomical source of

donor tissue, final location of the graft in the host, time after transplantation and the characteristics of the behavioral task, all played a part in determining the likelihood of functional benefits in our rats with hippocampal damage.

Our transplant survival rate of 85% may be higher than that normally predicted for subjects with a long interval (in our case 5 months) between the initial brain damage and the neural grafting procedure. Up to a point, delay between brain lesions and neural transplantation has been shown to significantly enhance the permanence of neural grafts[17]. However, Stein et al.[50] have reported that a 2 week delay between aspiration lesions and neural transplantation significantly reduces graft survival. These beneficial or injurious effects of delayed transplantation may depend on the waxing and waning of neurotrophic factors produced as a sequel to injury[18,40]. The extent of neurotrophic factor release in hippocampus following neonatal brain X-irradiation is currently unknown. It is possible however, that the surgical procedure involved in the stereotaxic placement of fetal nervous tissue into the adult brain may have provided sufficient stimulation of neurotrophic activity to promote the superior graft survival that we observed.

By eliminating the fascia dentata granule cells that normally receive massive afferents from the entorhinal cortex[42] it is possible to produce many of the functional deficits that normally characterize hippocampectomy[53]. Like the behavioral deficits observed after much larger hippocampal lesions, our discrete damage to the fascia dentata granule cells produced impaired learning of a passive avoidance task and perseverative spontaneous turning in a hemisphere apparatus (bowl). The T maze has been used frequently to assess deficits in spontaneous alternation and as an indicator of the fixated response patterns characteristic of animals with hippocampal lesions[36]. In an important way the plastic hemisphere we used is like a T maze. In a T maze a spontaneously alternating rat reverses direction on each discrete trial. In the bowl, there is no discrete choice point. When a rat has made a full circle in the plastic hemisphere it begins to retrace the path most recently traversed. This presents an opportunity to either continue or reverse direction. The mean turning bout length is essentially a measure of alternation frequency corrected for the total number of quarter turns made. Therefore mean bout length may be considered a continuous (non-discrete-trial) measure of spontaneous alternation.

Our experiments are the first to look at the behavioral changes that follow the placement of neural grafts in the brains of rats with discrete damage to the fascia dentata granule cells. However, others have used a model of hippocampal deafferentation (fimbria-fornix lesions) and reported either: (1) potentiation of impairments on a serial alternation task after intrahippocampal placement of large septal grafts[19], or (2) improvements in water maze[41] or T maze performance[21] after transplantation of smaller septal grafts into hippocampus. Additional studies have evaluated the functional recovery of rats following the placement of embryonic hippocampal transplants in the cavity remaining after large aspiration lesions of the hippocampal formation. Both maze learning[33] and performance of an operant task requiring low response rates[47] are improved by these grafting procedures.

Transplant-induced recovery was not observed simultaneously on our 2 behavioral tasks. During the first behavioral retest (almost 3 months after surgery) the passive avoidance performance of our transplanted rats was superior to that of control animals without grafts. However, at this same time, perseverative spontaneous turning was similar in all irradiated subjects irrespective of any transplant manipulations. Later, during the final behavioral test series, (approx. 6 months after surgery) irradiated rats with hippocampal grafts showed less perseverative turning than did subjects that did not receive these grafts. Graft-mediated functional benefits in passive avoidance acquisition were not apparent during this second retest. Others have also reported that grafts offer task-specific behavioral benefits to rats with fimbria-fornix lesions[21]. In addition, the functional consequences of embryonic neocortex transplanted in rats with prefrontal cortex lesions depends on the time after grafting that the behavioral measures are recorded[22]. Collectively, these data suggest that behavioral recovery, following placement of neural grafts in the brains of rats with fascia dentata damage, depends on the behavior being measured and the time after grafting that the measurement occurs. It may be noted that the benefits of our neural transplants were most apparent during test series when respective performances of irradiated and sham-irradiated control subjects (without grafts) were most divergent. It is possible that the timing of discernible behavioral recovery in grafted animals is not only dependent on progressive host/graft interactions but may also be due, in part, to changes in the performance of our controls over the course of the experiments.

In rats without transplants, perseverative movements (originally observed during behavioral baseline measures) became more severe during the course of the experiment (see also ref. 39). This raises the question whether our grafts produced a 'true' recovery of function or only prevented a time-related exacerbation of perseveration. However our findings suggest that the mean turning bout lengths of subjects with homotopic fascia dentata grafts (i.e. located within the hippocampal formation) and those with cerebral cortex grafts, located

outside the hippocampal formation, were (a) significantly less than X-irradiated controls without transplants and (b) no different from the movements of subjects that were sham-irradiated. Thus, these transplant procedures not only prevented the time-related potentiation of perseverative turning, there was also a return to a level of functioning that was similar to that of normal rats.

In embryonic and early postnatal fascia dentata grafts new neurons are likely to be formed after transplantation. These neurons represent the substantial postnatal neurogenesis of dentate granule cells observed in the normal dentate gyrus[1,9]. However, the formation and differentiation of granule cells in fascia dentata transplants take place under conditions in the neonatally irradiated host that are different from those present in the normal dentate gyrus. Zimmer and his colleagues[58,60] have shown that the brain compensates for early radiation-induced damage to the hippocampal granule cells by stimulating dendritic growth. Their results demonstrate that a reduction of the fascia dentata population can induce: (1) a compensatory increase in the neuropil layers containing the dendrites of the remaining neurons, (2) a corresponding relative increase in their axonal projections, and (3) a shift and expansion of afferent projections to an adjacent neuronal population.

These compensatory changes in the host brain raise a question regarding the extent that an abnormal host environment may affect the structural and functional characteristics of granule cell transplants. In this regard Zimmer et al.[58] have reported the superior ability of granule cell transplants to normalize dentate afferent and efferent connections when grafts are placed in the host brain immediately after the radiation-induced hippocampal damage. However, if the grafts are delayed from 5 to 10 weeks after the initial injury there is little ingrowth of hippocampodentate fibers into the transplants. These data suggest that it may be more difficult to demonstrate functional benefits in animals that have received neural transplants long after the initial brain injury. Indeed, a result consistent with this hypothesis has been reported after cortical transplants were placed in the adult brain 30–60 days following aspiration lesions of the cerebral cortex[50]. The current findings are especially provocative in the context of the data just discussed since we recorded improved performance in rats transplanted over 5 months after radiation exposure produced hypoplasia of the fascia dentata granule cells.

When our data are taken in conjunction with those of Zimmer et al.[58] the question may be raised about the unlikely role for neural reconnection in the behavioral recovery we observed. Similarly, others have suggested that specific neural connections between host and transplant may not be necessary for behavioral recovery[24,25,50]. Instead, the grafts may produce needed neurotransmitters[23] or provide endogenous brain-specific trophic factors that can rescue the damaged host brain or restore function[10,14,17,18,27,48]. Unfortunately the current data do not address these questions. The production of humoral or neurotrophic factors in subjects with early radiation-induced granule cell hypoplasia (the relatively 'non-traumatic' injury used here) has yet to be demonstrated. We have experiments in progress that will assess host/graft neural interconnections in our subjects. Additional studies are being analyzed that have looked at the behaviorai recovery of rats with fascia dentata granule cell hypoplasia after earlier placement of hippocampal transplants when potential host/graft interactions are more probable (within a month of the initial injury).

Graft location and source are other factors that may be relevant to the prediction of behavioral recovery following brain injury. Although we attempted to place all our fetal tissue grafts within the hippocampal formation, our histological review revealed that pieces of graft were often outside of this structure. Our behavioral analysis showed that cerebral cortex transplants located within the hippocampal formation neither improved indicators of passive avoidance performance nor reduced bouts of perseverative turning. However, if the cortical cells were located outside of the hippocampus (usually in cerebral cortex or lateral ventricle), behavioral benefits were observed. Further, fascia dentata grafts were generally most effective when they were found to be located in the hippocampal formation of brain damaged rats. The literature suggests that a variety of behavioral results can follow the transplantation of nonhomologous neurons. Animals with homologous tissue grafts frequently exhibit post-lesion behavioral recovery superior to that observed after nonhomologous brain tissue transplants[37]. Woodruff et al.[57] report, for example, that rats with hippocampal tissue grafts transplanted into a hippocampal lesion site, performed better on an operant task requiring slow response rates than did subjects receiving nonhomologous grafts of fetal hindbrain tissue. However, functional benefits of nonhomologous neural grafts have also been observed[49]. Further, reports indicate that a neural graft survives best when transplanted into its corresponding region of origin in the host brain[28]. The importance of graft location has been emphasized by Sunde et al.[52] who suggested that 'misplacement' of hippocampal transplants could result in abnormal serial connections in a brain with fascia dentata granule cell hypoplasia. In the current study we found a possible behavioral analogue of this neuroanatomical observation. To optimize behavioral recovery from radiation-induced hypoplasia of hippocampal granule cells, fascia dentata grafts must be placed within the hippocampal formation.

Further, 'misplacement' of cerebral cortex transplants into the host hippocampus failed to improve performance and enhanced behavioral variability (see Fig. 3).

It may be of interest to speculate that our partial success in using cerebral cortex grafts to reduce behavioral decrements following fascia dentata granule cell hypoplasia may be due, in part, to the similar anlage of neocortical and hippocampal tissue[2]. In this regard, Stein et al.[49] have reported that functional deficits following bilateral lesions of the occipital cortex could be reduced by frontal cortex transplants having similar developmental origins as the tissue removed. Conversely, Woodruff et al.[57] have shown that neural transplants derived from a different anlage (the hindbrain) have proven to be ineffective in reversing behavioral deficits associated with hippocampal lesions. These data suggest that there may be some gradient of homology that permits cortical tissue more restorative influence than hindbrain tissue in the recovery of brain function following damage to the hippocampus.

In summary, we found that the behavioral deficits associated with radiation-induced hypoplasia of the neonatal fascia dentata granule cells can be diminished by transplanting fetal brain tissue into the damaged adult brain. The time course of transplant-induced behavioral recovery was not uniform for all behaviors measured. Although performance was improved following cerebral cortex transplantation, fascia dentata grafts were generally more effective than cortical grafts in promoting behavioral recovery. We also found that behavioral recovery can be promoted by locating the transplanted tissue in the host nuclei that corresponds to the neuroanatomical source of the donor tissue. The nonhomotopic placement of grafts (e.g. cortex into hippocampus) failed to produce these behavioral benefits. Collectively these data suggest important functional benefits following the transplantation of fetal hippocampal tissue into the brains of adult rats that experienced radiation-induced hypoplasia of fascia dentata granule cells as neonates. This functional recovery is optimized following a precise combination of experimental conditions that take into acount behavioral task requirements, time course of recovery, and the neuroanatomical source and final location of transplanted neurons.

Acknowledgements. We thank Dr. Paul Reier and Dr. Herman Teitelbaum who contributed to the conceptualization of these experiments. We also appreciate the especially helpful comments offered by the peer reviewers of this article. The authors recognize the excellent technical assistance provided by Mr. Mark Postler, Ms. Diane Pickle and Dr. Cathie Akkerks. The dosimetry and irradiations were performed by Mr. V. Scott Hawkins and Mr. Ernest Golightly. Statistical advice was provided by Mr. William Jackson. We thank Ms. Lilly Heman-Ackah for her excellent histological assistance. This research was supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under Work Unit 00163. Views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred. Research was conducted according to the principles enunciated in the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources, National Research Council.

REFERENCES

1 Altman, J. and Das, G.D., Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats, *J. Comp. Neurol.*, 124 (1965) 319–336.

2 Angevine, J.B., Development of the hippocampal region. In R.L. Isaacson and K.H. Pribram (Eds.), *The Hippocampus, Vol. 1 Structure and Development*, Plenum, New York, 1975, pp. 61–94.

3 Azmitia, E.C. and Björklund, A., Cell and tissue transplantation into the adult brain, *Ann. N.Y. Acad. Sci.*, 495 (1987) 1–813.

4 Banks, W.J., *Histology and Comparative Organology: A Text Atlas*, Williams and Wilkins, Baltimore, 1974.

5 Bayer, S.A., Brunner, R.L., Hine, R. and Altman, J., Behavioural effects of interference with the postnatal acquisition of hippocampal granule cells, *Nature New Biol.*, 242 (1973) 222–224.

6 Bayer, S.A. and Altman, J., Hippocampal development in the rat: cytogenesis and morphogenesis examined with autoradiography and low-level x-irradiation, *J. Comp. Neurol.*, 158 (1974) 55–80.

7 Bayer, S.A. and Altman, J., Radiation-induced interference with postnatal hippocampal cytogenesis in rats and its long-term effects on the acquisition of neurons and glia, *J. Comp. Neurol.*, 163 (1975) 1–20.

8 Bayer, S.A. and Peters, P.J., A method for X-irradiating selected brain regions in infant rats, *Brain Res. Bull.*, 2 (1977) 153–156.

9 Bayer, S.A., Development of the hippocampal region in the rat. I. Neurogenesis examined with ^{3}H-Thymidine autoradiography, *J. Comp. Neurol.*, 190 (1980) 87–114.

10 Bernstein, J.J. and Goldberg, W.J., Fetal spinal cord homografts ameliorate the severity of lesion-induced hind limb behavior deficits, *Exp. Neurol.*, 98 (1987) 633–644.

11 Björklund, A. and Stenevi, U., Reformation of the severed septohippocampal cholinergic pathway in the adult rat by transplanted septal neurons, *Cell Tissue Res.*, 185 (1977) 289–302.

12 Björklund, A. and Stenevi, U., Intracerebral neural implants: neuronal replacement and reconstruction of damaged circuitries, *Annu. Rev. Neurosci.*, 7 (1984) 279–308.

13 Björklund, A. and Stenevi, U., *Neural Grafting in the Mammalian CNS*, Elsevier, Amsterdam, 1985, pp. 3–701.

14 Bregman, B.S. and Reier, P.J., Neural tissue transplants rescue axotomized rubrospinal cells from retrograde death, *J. Comp. Neurol.*, 224 (1986) 86–95.

15 Brunner, R.L., A cross-sectional study of behavior at three ages after neonatal X irradiation of the hippocampus, *Behav. Biol.*, 22 (1978) 211–218.

16 Casarett, G.W., *Radiation Histopathology, Vol. II*, CRC Press, Boca Raton, 1980.

17 Cotman, C.W., Nieto-Sampedro, M. and Whittemore, S.R., Relationships between neurotrophic factors and transplant-host integration. In A. Björklund and U. Stenevi (Eds.), *Neural Grafting in the Mammalian CNS*, Elsevier, Amsterdam, 1985, pp. 169–178.

18 Cotman, C.W. and Kesslack, P., The role of trophic factors in behavioral recovery and integration of transplants. In D.M. Gash and J.R. Sladek (Eds.), *Transplantation into the Mamma-*

lian CNS, Progress in Brain Research, Vol. 78, Chapter 39, Elsevier, Amsterdam, 1988, pp. 311–319.
19 Dalrymple-Alford, J.C., Kelche, C., Cassel, J.C., Toniolo, G., Pallage, V. and Will, B.E., Behavioral deficits after intrahippocampal fetal septal grafts in rats with selective fimbria-fornix lesions, *Exp. Brain Res.*, 69 (1988) 545–558.
20 Douglas, R.J., The hippocampus and behavior, *Psychol. Bull.*, 67 (1967) 416–442.
21 Dunnett, S.B., Low, W.C., Iversen S.D., Stenevi, U. and Björklund, A., Septal transplants restore maze learning in rats with fornix-fimbria lesions, *Brain Research*, 251 (1982) 335–348.
22 Dunnett, S.B., Ryan, C.N., Levin, P.D., Reynolds, M. and Bunch, S.T., Functional consequences of embryonic neocortex transplanted to rats with prefrontal cortex lesions, *Behav. Neurosci.*, 101 (1987) 489–503.
23 Freed, W.J., Olson, L., Ko, G.N., Morihisa, J.M., Niehoff, D., Stromberg, I., Kuhar, M., Hoffer, B.J. and Wyatt, R.J., Intraventricular substantia nigra and adrenal medulla grafts: mechanisms of action and ^{3}H-Spiroperidol autoradiography. In A. Björklund and U. Stenevi (Eds.), *Neural Grafting in the Mammalian CNS*, Elsevier, Amsterdam, 1985, pp. 471–489.
24 Gage, F.H., Björklund, A., Stenevi, U., Dunnett, S.B. and Kelly, P.A.T., Intrahippocampal septal grafts ameliorate learning impairments in aged rats, *Science*, 225 (1984) 533–536.
25 Giordano, M., Hagenmeyer-Houser, S.H. and Sanberg, P.R., Intraparenchymal fetal striatal transplants and recovery in kainic acid lesioned rats, *Brain Research*, 446 (1988) 183–188.
26 Greenstein, S. and Glick, S.D., Improved automated apparatus for recording rotation (circling behavior) in rats or mice, *Pharmacol. Biochem. Behav.*, 3 (1975) 507–510.
27 Haun, F. and Cunningham, T.J., Rescuing damaged lateral geniculate neurons with neural transplants, *Anat. Rec.*, 205 (1983) 77A.
28 Heuschling, P., De Paermentier, F. and van den Bosch de Aguilar, P., Topographical distribution in the adult rat brain of neurotrophic activities directed to central nervous system targets, *Dev. Brain Res.*, 38 (1988) 9–17.
29 Hicks, S.P., Radiation as an experimental tool in mammalian developmental neurology, *Physiol. Rev.*, 38 (1958) 337–356.
30 Hicks, S.P. and D'Amato, C.J., Effects of ionizing radiations on mammalian development. In D.H.M. Woollam (Ed.), *Advances in Teratology*, Logos Press, London, 1966, pp. 195–250.
31 Kimble, D.P., Hippocampus and internal inhibition, *Psychol. Bull.*, 70 (1968) 285–295.
32 Kimble, D.P., Bremiller, R. and Stickrod, G., Fetal brain implants improve maze performance in hippocampal-lesioned rats, *Brain Research*, 363 (1986) 356–363.
33 Laatsch, R.H. and Cowan, W.M., Electron microscope studies of the dentate gyrus of the rat. I. Normal structure with special reference to synaptic organization, *J. Comp. Neurol.*, 128 (1966) 359–395.
34 LaBossiere, E., *Histological Processing for the Neural Sciences*, Thomas, Springfield, 1976.
35 Low, W.C., Lewis, P.R., Bunch, S.T., Dunnett, S.B., Thomas, S.R., Iversen, S.D., Björklund, A. and Stenevi, U., Function recovery following neural transplantation of embryonic septal nuclei in adult rats with septohippocampal lesions, *Nature (Lond.)*, 300 (1982) 260–262.
36 Means, L.W., Leander, J.D. and Isaacson, R.L., The effects of hippocampectomy on alternation behavior and response to novelty, *Physiol. Behav.*, 6 (1971) 17–22.
37 Mickley, G.A., Teitelbaum, H. and Reier, P.J., Fetal hypothalamic brain grafts reduce the obesity produced by ventromedial hypothalamic lesions, *Brain Research*, 424 (1987) 239–248.
38 Mickley, G.A., Ferguson, J.L., Nemeth, T.J., Mulvihill, M.A. and Alderks, C.E., Spontaneous perseverative turning in rats with radiation-induced hippocampal damage, *Behav. Neurosci.*, 103 (1989) 722–730.
39 Mickley, G.A., Ferguson, J.L., Mulvihill, M.A. and Nemeth, T.J., Progressive behavioral changes during the maturation of rats with early radiation-induced hypoplasia of fascia dentata granule cells, *Neurotoxicol. Teratol.*, 11 (1989) 385–393.
40 Nieto-Sampedro, M., Whittemore, S.R., Needelt, D.V., Larson, J. and Cotman, C.W., The survival of brain transplants is enhanced by extracts from injured brain, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 6250–6254.
41 Nilsson, O.G., Shapiro, M.L., Gage, F.H., Olton, D.S. and Björklund, A., Spatial learning and memory following fimbria-fornix transection and grafting of fetal septal neurons to the hippocampus, *Exp. Brain Res.*, 67 (1987) 195–215.
42 O'Keefe, J. and Nadel, L., *The Hippocampus as a Cognitive Map*, Oxford University Press, Oxford, 1978, pp. 102–140.
43 Olton, D.S., Memory functions and the hippocampus. In W. Seifert (Ed.), *Neurobiology of the Hippocampus*, Academic Press, London, 1983, pp. 335–373.
44 Paxinos, G. and Watson, C., *The Rat Brain in Stereotaxic Coordinates*, Academic Press, Sydney, 1982.
45 Seress, L. and Pokorny, J., Structure of the granular layer of the rat dentate gyrus, *J. Anat.*, 133 (1981) 181–195.
46 Sladek, J.R. and Gash, D.M. (Eds.), *Neural Transplants — Development and Function*, Plenum Press, New York, 1984, pp. 1–443.
47 Snedecor, G.W. and Cochran, W.G., *Statistical Methods*, 7th edn., Iowa State University Press, Ames, Iowa, 1980, pp. 96–98.
48 Stein, D.G., Labbe, R., Firl, A. and Mufson, E.J., Behavioral recovery following implantation of fetal brain tissue into mature rats with bilateral, cortical lesions. In A. Björklund and U. Stenevi (Edt.), *Neural Grafting in the Mammalian CNS*, Elsevier, Amsterdam, 1985, pp. 605–614.
49 Stein, D.G., Labbe, R., Attella, M.J. and Rakowsky, H.A., Fetal brain tissue transplants reduce visual deficits in adult rats with bilateral lesions of the occipital cortex, *Behav. Neural Biol.*, 44 (1985) 266–277.
50 Stein, D.G., Palatucci, C., Kahn, D. and Labbe, R., Temporal factors influence recovery of function after embryonic brain tissue transplants in adult rats with frontal cortex lesions, *Behav. Neurosci.*, 102 (1988) 260–267.
51 Stenevi, U., Kromer, L.F., Gage, F.H. and Björklund, A., Solid neural grafts in intracerebral transplantation cavities. In A. Björklund and U. Stenevi (Eds.), *Neural Grafting in the Mammalian CNS*, Elsevier, Amsterdam, 1985, pp. 41–49.
52 Sunde, N., Laurberg, S. and Zimmer, J., Brain grafts can restore irradiation-damaged neuronal connections in newborn rats, *Nature (Lond.)*, 310 (1984) 51–53.
53 Wallace, R.B., Kaplan, R.F. and Werboff, J., Behavioral correlates of focal hippocampal X-irradiation in rats, *Exp. Brain Res.*, 24 (1976) 343–349.
54 Wallace, R.B., Graziadei, R. and Werboff, J., Behavioral correlates of focal hippocampal X-irradiation in rats, II, *Exp. Brain Res.*, 43 (1981) 207–212.
55 Weisbroth, S.H., Bacterial and mycotic diseases. In H.J. Baker, J.R. Lindsey and S.H. Weisbroth (Eds.), *The Laboratory Rat, Vol. I. Biology and Diseases*, Academic Press, New York, 1979, pp. 206–208.
56 Winer, B.J., *Statistical Principles in Experimental Design*, McGraw-Hill, New York, 1971, pp. 30–35, 149–260, 397–402.
57 Woodruff, M.L., Baisden, R.H., Whittington, D.L. and Benson, A.E., Embryonic hippocampal grafts ameliorate the deficit in DRL acquisition produced by hippocampectomy, *Brain Research*, 408 (1987) 97–117.
58 Zimmer, J., Sunde, N. and Sorensen, T., Reorganization and restoration of central nervous connections after injury: a lesion and transplant study of the rat hippocampus. In B.E. Will, P. Schmitt and J.C. Dalrymple-Alford (Eds.), *Brain Plasticity, Learning and Memory*, Plenum, London, 1985, pp. 505–518.
59 Zimmer, J., Laurberg, S. and Sunde, N., Non-cholinergic afferents determine the distribution of the cholinergic septohippocampal projection: a study of the AChE staining pattern in the rat fascia dentata and hippocampus after lesions, X-irradiation, and intracerebral grafting, *Exp. Brain Res.*, 64 (1986) 158–168.
60 Zimmer, J., Poulsen, P.H., Blackstad, J.W. and West, M.J., Radiation-induced reduction of the rat dentate gyrus. Compensatory cellular and connectional changes, *Soc. Neurosci. Abstr.*, 13 (1987) 1602.

INT. J. RADIAT. BIOL., 1990, VOL. 57, NO. 2, 437–442

Conference report

The First Consensus Development Conference on the Treatment of Radiation Injuries

The First Consensus Development Conference on the Treatment of Radiation Injuries sponsored by the Medical Radiobiology Advisory Team (MRAT) of the Armed Forces Radiobiology Research Institute (AFRRI) was held in Washington, DC, on 10–13 May 1989. Physicians and scientists from around the world met to discuss the most appropriate treatment(s) for the haematopoietic and infectious complications that accompany radiation injuries and for combined radiation and traumatic/burn injuries.

The three most recent accidents [the reactor explosion in Chernobyl, USSR (1986); the internal and external exposure to caesium-137 in Goiania, Brazil (1987); and the occupational exposure in a radiation sterilization facility in San Salvador, El Salvador (1989)] show that in radiation accidents the exposure environment is likely to be ill defined and uncontrolled. Physicians treating irradiated patients may find that the patients also have been traumatized by burns and/or wounds (*i.e.* combined injuries).

The immediate treatment of associated injuries is a primary determinant of survival. The nature of the radiation-induced marrow aplasia (reversible or irreversible) may not be known for days, because the dose of radiation may vary in rate and quality, and the exposure probably will not be uniform or homogeneous (*i.e.* involve only part of the body). These facts make reliance on a physical dose estimate impossible, and underscore the need for monitoring biological parameters to estimate the severity of injuries and the probability of survival. Reliable triage and good clinical care, based on comprehensive biological data, will ensure the best possible chance for casualty recovery should a critical number of stem cells survive the radiation exposure.

Data for uncomplicated human radiation exposures within the haematopoietic syndrome range are relatively limited. The evidence (exclusive of the three recent accidents) comes from four primary sources: (1) persons exposed to the nuclear weapons detonations in Hiroshima and Nagasaki, (2) radiotherapy of persons with Ewing's sarcoma with therapeutic total-body, bilateral exposure to γ radiation, (3) two nuclear criticality accidents (Vinca, Yugoslavia, and Oak Ridge, Tennessee) involving subjects exposed to mixed neutron and gamma radiation, and (4) bone marrow transplant recipients receiving total-body γ radiation. Information collected from these exposures, the three recent accidents and experimental data has provided us with the basis for establishing a consensus on the treatment of radiation injuries (figure 1).

Supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency. Views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred.

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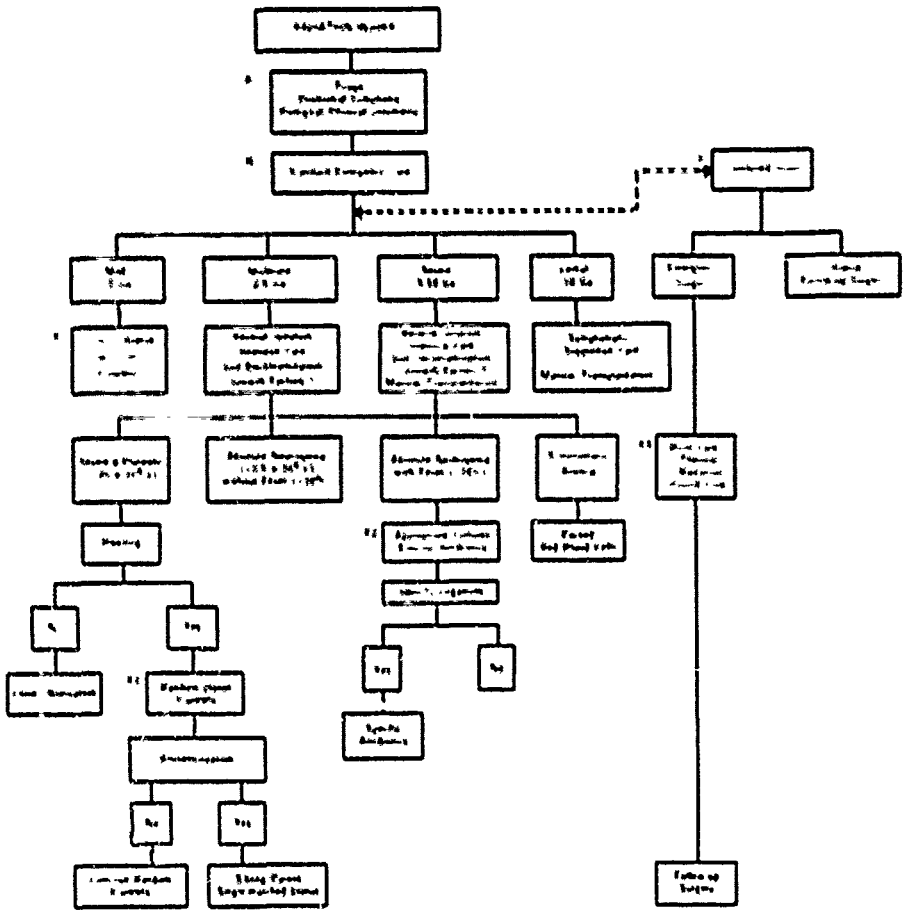

Figure 1. *Treatment schema.* The outlined protocol for treatment of radiation injuries is based on uniform total-body radiation exposure (free-in-air), and may not be applicable in all accident scenarios or mass casualty situations.

Triage (figure 1, A)

Estimating the degree of radiation damage and exposure is difficult. Prodrome, which begins within hours of exposure, is characterized by gastrointestinal and neurovascular signs and symptoms, including nausea, vomiting, diarrhea, fatigue, weakness, fever and headache. The time of occurrence, severity and duration of these signs are dose and dose-rate dependent, and must be used in conjunction with early biological parameters, such as peripheral lymphocyte counts, to determine the presence and severity of acute radiation syndrome. These signs and symptoms must be used initially to determine if the casualty is one of radiation exposure.

The rate and degree of loss of blood cellular elements are dose dependent. Blood samples should be taken daily during the first week of treatment. A useful rule of thumb is, if lymphocytes have decreased by 50 per cent and are less than 1×10^9/l within 24–48 h, the patient has received at least moderate radiation exposure. In the event of combined injuries, lymphocytes may be an unreliable indicator. Patients

who have received severe burns and/or trauma to more than one system often develop lymphopenia.

Effective triage relies on accurate analysis of the early signs and symptoms, substantiated by biological parameters associated with radiation exposure. Treatment must be determined after analysing the radiation injury and indicated therapies for the type of organ damage. Conventional injuries that are benign by themselves can become lethal when combined with radiation exposure.

Emergency care (figure 1, B)

Because radiation injury is not immediately life-threatening, initial care should address the associated conventional injuries, *e.g.* thermal burns and wounds. The standard emergency medical procedures for ventilation, perfusion and haemorrhage should be provided first, and then casualties should be stabilized. After stabilization, decontamination and treatment of the radiation injuries should continue.

Combined injury (figure 1, C)

During triage, associated injuries (trauma/burn) should be assessed by standard procedures, keeping in mind that the signs and symptoms of associated injuries can mimic and obscure those caused by acute radiation effects. Experimental data indicate that the combination of conventional trauma and radiation injury has adverse synergistic effects. Several points in the medical management of combined injury casualties are important. For example, the optimum period during which surgery can be performed successfully is different from that of conventional trauma. Surgical correction of life-threatening and other major injuries should be carried out as soon as possible (within 36–48 h), while elective procedures should be postponed until late in the convalescent period (45–60 days).

Radiation categories (figure 1, D)

Based on the severity of radiation exposure, casualties can be classified into four treatment categories: mild, moderate, severe and lethal. A consensus was not reached on the specific dose ranges (free-in-air) for these categories, primarily because of the difficulty in converting an air dose to a meaningful tissue dose. Nevertheless, dose ranges are indicated in figure 1. The main goal of this classification system is to predict the likelihood of survival of the casualty's haematopoietic stem cells. In treating the casualty, and not the radiation dose, the most reliable guide for the physician is the change in blood elements resulting from bone marrow injury.

Initial care (figure 1, E)

Medical treatment of casualties with moderate and severe radiation exposure should include early institution of reverse isolation. Prophylactic use of selective gut decontamination with oral nonabsorbable antibiotics and avoidance of uncooked food can be useful. These measures help control the gastrointestinal source of post-injury infections. Maintenance of gastric acidity (*i.e.* avoidance of antacids and H_2 blockers) may prevent bacteria from entering through the gastric mucosa. When possible, early oral feeding is preferred to intravenous feeding to maintain the immunological and physiological integrity of the gut. Surgical implantation of a subcutaneously tunnelled central venous catheter should be considered to allow

frequent venous access. However, excessive use of catheters can predispose the patient to bacteraemia.

Irreversible damage to the bone marrow stem cells, in a dose-dependent pattern, is a complication of total-body radiation exposure. The dose range that would deplete the entire stem cell pool is still unknown. However, based on animal experiments, using a uniform tissue dose throughout the body, it appears that bone marrow transplantation from a histocompatibility locus antigen (HLA) identical donor may be beneficial at doses of uniform total-body irradiation as low as 5 Gy. Even a transient engraftment for 3–7 weeks may be helpful to casualties exposed to uniform whole-body radiation of 5–6 Gy during pancytopenia, until autologous stem cell recovery occurs.

The immediate post-exposure period is a time of profound immunosuppression and can be exploited as a preparatory phase for marrow transplantation for casualties of moderate to severe doses of radiation. This will influence the physician's decision on separate immunosuppressive regimens. Therefore, early in the treatment of this group, suitable donors need to be identified for HLA typing and mixed lymphocyte culture studies. The most appropriate time for marrow transplantation is still uncertain, because the reversibility of haematopoietic damage is not known during initial care. However, the ideal time appears to be within 3–5 days post-exposure, based on animal data.

Growth factors such as recombinant human granulocyte and granulocyte–macrophage colony stimulating factors (rhG-CSF and rhGM-CSF) are potent stimulators of haematopoiesis. Casualties with moderate to severe exposure would most likely benefit from therapeutic use of haematopoietic growth factors. Preclinical studies in larger animals and Phase I and II clinical trials in patients with bone marrow aplasia, resulting from chemotherapy, therapeutic total-body radiation or AIDS, have established the efficacy and safety of rhG-CSF and rhGM-CSF in enhancing granulocyte recovery. Use of rhG-CSF or rhGM-CSF is appropriate in casualties likely to have prolonged severe granulocytopenia. Although the dose range is uncertain, a reasonable approximation may be uniform total-body exposure of 4–8 Gy. Growth factors may have to be initiated early in the treatment to be effective at the right time.

Early granulocyte recovery, following growth factor therapy, may indicate a lower radiation dose exposure. While growth factors may benefit some patients, the type of radiation exposure is crucial in assessing the long-term sequelae of therapy. Prolonged internal irradiation from inhaled or ingested materials may be a contraindication for the use of growth factors. Potential risks include acceleration of leukemogenesis. Pharmacologic doses of rhGM-CSF may adversely affect neutrophil migration into soft tissues, an issue of considerable importance in the treatment of combined injury.

Definitive care

Transfusion (figure 1, F1)

The requirements for platelet support depend on the condition of the patient. The threshold for instituting platelet support should be at 20×10^9/l, unless other major medical problems or bleeding are present. Platelets are likely to come from random donors. Should refractoriness develop, family members and HLA-compatible donors from the community can be considered. Gamma radiation of blood products with 15–20 Gy is recommended to abolish the mitotic activity of

lymphocytes without adversely affecting the red blood cells, platelets and plasma proteins.

Antibiotics (figure 1, F2)

Management of established or suspected infection (neutropenia and fever) in irradiated casualties is similar to that used in other febrile neutropenic patients. An empirical regimen of antibiotics should be selected, based on the severity of granulocytopenia and the patterns of bacterial susceptibility and nosocomial infections present in the institution. Combination antibiotic therapy is recommended, or monotherapy (*i.e.* ceftaxidime or imipenem) as appropriate. Modification of this initial antibiotic regimen should include a thorough evaluation of the history, physical examination, epidemiological information and laboratory data. Antifungal (amphotericin B) coverage should be added for patients who remain persistently febrile for 7 or more days or who have new fever on or after the 7th day of antibiotics. If there is evidence of resistant gram-positive infection, vancomycin should be added. Surveillance cultures may be useful for monitoring resistant bacteria and emergence of fungi. Twice-weekly sampling or surveillance cultures would be reasonable. This regimen should be modified depending on the institutional patterns of nosocomial infections.

Burn and wound care (figure 1, F3)

Treatment of thermal burns should include early excision of potentially septic tissues and closure of the wounds, preferably by skin grafting. Radiation and thermal burns should be treated differently, especially when using surgery, which should be delayed in the case of radiation burns. Information on the specific treatment of radiation burns to the skin is currently not available.

The closure of the traumatic wound, which generally should be attempted as soon as possible, emerges as the most challenging of all surgical therapeutic efforts in the treatment of the combined-injury patient. This occurs because of the relatively short period following injury when surgery can be performed safely. This narrow time-frame results from radiation-induced suppression of cellular elements necessary for wound healing and prevention of infection and haemorrhage. Experimental data indicate increased mortality in the absence of wound closure. The following procedures are recommended after resuscitation and emergency surgery: (1) the patient should be returned to the operating room within 48 h, obtain quantitative cultures and, if the wound is clinically clean, graft all defects with autologous skin; (2) remove dressings at 96–120 h and, if the wounds were appropriately debrided, use skin grafts to close the wounds; and (3) if additional debridement is necessary at 48 h, the previous procedures should begin again at 96 h.

The proceedings of the conference, including more detailed consensus statements, discussions and invited papers will be published by Plenum Press, New York (*Treatment of Radiation Injuries*, ed. by D. Browne, J. F. Weiss, T. J. MacVittie and M. V. Pillai). The consensus panel members are listed below.

Hematopoietic Injury Complications: Eugene P. Cronkite, MD (moderator); Rainer Storb, MD; Richard Champlin, MD; C. Robert Valeri, MD; Joseph Laver, MD; Thomas J. MacVittie, PhD; Joseph H. Antin, MD; Robert P. Gale, MD; and Dorothee Krumwieh, MD.

Infectious Complications of Radiation Injury: Richard I. Walker, PhD and Itzhak Brook, MD (moderators); Stephen C. Schimpff, MD; Alexandre R. de Oliveira, MD; Gary P. Zaloga, MD; Thomas Walsh, MD; and Anna Butturini, MD.
Combined Injury Complications: Robert W. Young, PhD (moderator); Erwin Hirsch, MD; William I. Becker, MD; Patricia Mertz; G. David Ledney, PhD; and Robert C. Ricks, PhD.

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